Brush border digestion:
Development of a physiologically relevant *in vitro* model.

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Diane Frances Hooton
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Abstract

The majority of current *in vitro* digestion methods either exclude the small intestinal brush border (BB) phase of digestion or do not incorporate the entire array of BB enzymes that are required to achieve terminal endogenous digestion *in vivo*. Accordingly, the digestate, and its derivitives, may not be representative of the digestive process *in vivo*. In order to improve the fidelity of the *in vitro* digestion process this thesis developed a physiologically relevant small intestinal BB phase using enzymes isolated from rat small intestinal mucosal tissue. The activities of BB enzymes were assessed and compared with known values, and under conditions physiologically representative of the small intestine. Although there were significant differences in BB enzyme activities depending on pH, enzyme solubilisation, and upon prolonged exposure to biliopancreatic secretions the BB preparation was deemed suitable for use in an *in vitro* digestion method.

A rationale for the composition of the BB digestive phase was developed based on published physiological data, and was validated using glycosylated polyphenolic compounds as substrates. Liquid chromatography mass spectrometry (LC-MS) was used to assess the derivatisation products of BB digestion. In the absence of biliopancreatic secretions the onion flesh polyphenolic compounds quercetin-4′-glucoside and isorhamnetin-4′-glucoside, but not quercetin-3-glucoside or quercetin-34′-diglucoside were hydrolysed. The positive control quercetin-3-glucoside was hydrolysed, and the negative control quercetin-3-rutinoside was not hydrolysed. The deglycosylation of quercetin-3-glucoside was monitored under conditions representative of the small intestine, *i.e.* incorporating bile and pancreatin, while at the appropriate pH. Quercetin-3-glucoside was significantly deglycosylated in BB treatments (no treatment or pancreatin alone) compared to BB treatments with bile (bile alone or pancreatin and bile).

The mammalian digestive system is equipped to hydrolyse macronutrients from their polymeric form through to monomers and oligomers suitable for absorption across the epithelial layer. As such the inactivation or degradation of some BB enzymes during the BB digestive phase by bile or pancreatin was not unexpected, and does not preclude its use as an *in vitro* tool in the future.

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List of acronyms

Acronym	Protein name	Description / Function
ACE	Angiotensin converting enzyme	Brush border peptidase, peptide hormone
ALP	Alkaline phosphatase	Hydrolyzes phosphate residues, regulatory
APA	Aminopeptidase A	Brush border peptidase
APN	Aminopeptidase N	Brush border peptidase
APP	Aminopeptidase P	Brush border peptidase
AS	Alkaline sphingomyelinase	Sphingolipid hydrolysis
BB	Brush border	Intestinal microvillar membrane
BBMV	Brush border membrane vesicle	Vesicle of microvillar membrane shed from the tips of microvillli
BSAL	Bile salt activated lipase	Lipase/Sterol esterase
CPA	Carboxypeptidase A	*C*-terminal pancreatic peptidase
CPB	carboxypeptidase B	*C*-terminal pancreatic peptidase
DP1	Dipeptidase 1	Brush border dipeptidase
DPPIV	Dipeptidylpeptidase IV	*N*-terminal dipeptidase
GGT	γ-glutamyl transpeptidase	Brush border peptidase
GPI	Glycophosphatidylinositol	Covalently attached glycolipid that anchors the protein to the membrane
LPH	Lactase-phlorizin hydrolase	Enzyme complex: *β*-glucosidase/glycosylceramidase
MEP	Meprin A subunit β	Brush border endopeptidase
MGAM	Maltase–glucoamylase	Enzyme complex: *α*-1,4-glucosidase
NEP	Neprilysin	Brush border endopeptidase
NC	Neutral ceramidase	Sphingolipid hydrolysis
NTC	Sodium taurocholate	Bile salt
PTL	Pancreatic triacylglycerol lipase	Lipase
PLA2	Phospholipase A2	Phospholipid hydrolysis
PLB1	Phospholipase B1	Phospholipid hydrolysis
RER	Rough endoplasmic reticulum	Cytosolic organelle
SC	Soluble cytosolic	Cytosolic enzyme/protein that is soluble
SI	Sucrase-isomaltase	Enzyme complex: *α*-1,4 and *α*-1-6-glucosidase

1 Chapter 1. Introduction: approaches to *in vitro* digestion

Understanding the complex physiology surrounding the digestive process is important when trying to mimic digestion experimentally. Many methods of *in vitro* digestion mimic digestion using only progressive changes in temperature, pH and physical biochemistry. Some methods have developed sophisticated equipment to simulate gastrointestinal motility, and use dialysis membranes and immortal cell lines in an attempt to emulate the absorptive process at a cellular level, but often the underlying physiology is only partially addressed. The process of digestion can be partially replicated by the simple admixture of digestive secretions and digestate, but factors such as the type and degree of mechanical force exerted on digesta and the rheology of digestive contents are often poorly reproduced. Further, emulating temporal, physical and biochemical changes in the composition of digesta during gastrointestinal residence is extremely difficult because digestion is modulated by complex processes, some of which are governed by biochemical feedback. Since the complexities of digestion are extremely difficult to replicate *in vitro* having knowledge of physiology is vital when designing a system of *in vitro* digestion. To design an *in vitro* digestion system, that replicates the entire digestive process, requires an understanding of the kinetics of digestion from the mouth to the terminal ileum. Such an understanding may allow current methods used for *in vitro* digestion to be brought closer in line with the physiological process of digestion. Such is the aim of this thesis.

1.1 The digestive process

The endogenous digestive process ends when nutrient oligomers are reduced to their component monomers, and are absorbed. This action occurs in successive compartments due to the combined action of buccal, gastric and small intestinal digestive secretions, which is supported by the muscular contractions of the gastrointestinal tract that facilitate mixing. Basic physiological data pertaining to these segments are listed in table 1.

1.1.1 Oral phase of digestion

During the oral phase of digestion particles of food are mechanically broken down and mixed with salivary secretions which contain the digestive enzymes salivary α-amylase and lingual lipase (Chen, 2009). Mucin glycoproteins, water and electrolytes present in salivary secretions help to moisten and agglomerate food particles into a cohesive bolus that can be readily and safely swallowed (Pedersen et al., 2002). The digestion of fat begins in the mouth with the action of lingual lipase. Lingual lipase is thought to contribute primarily to the oro-sensory perception of dietary lipids rather than be of major importance in digestion (Gambareli et al., 2007). Salivary α-amylase initiates the digestion of dietary carbohydrates (Chen, 2009). At first sight salivary amylase could be thought to contribute only superficially to the digestion of carbohydrates; as it is thought to readily lose amylolytic activity in the acidic milieu of the stomach (Pedersen et al., 2002), *i.e*. at a pH $\leq$ 3 (Rosenblum et al., 1988). However, magnetic resonance imaging suggests that a moderately viscous (guar gum) food bolus can remain intact in the stomach, for greater than an hour (Marciani et al., 2001b). This suggests that buccal enzymes may remain active within the confines of the bolus allowing digestion to continue, the degree of which is not known.

1.1.2 Gastric phase of digestion

The stomach is primed for the arrival of food during the cephalic phase, when the thought, sight or smell of food initiates the secretion of gastric juices in preparation for the gastric phase (Feldman and Richardson, 1986). The gastric phase of digestion has three components. Firstly, gastric parietal cells secrete hydrochloric acid into the gastric lumen, which facilitates the acid hydrolysis and denaturation of the food matrix (Hinsberger and Sandhu, 2004), and mediates the acid activation of the gastric pro-enzyme pepsinogen (Richter et al., 1998). Secondly, nutrient hydrolysis is facilitated by enzymes such as gastric lipase (Gargouri et al., 1986) and pepsin isoforms (activated pepsinogens) (Roberts et al., 2007). Thirdly, the contractile activity of the stomach facilitates the trituration (Lentle and Janssen, 2011b) and emulsification (Golding and Wooster, 2010) of food particles and nutrients respectively.

The stomach is a muscular sack that receives and stores digestate, reduces the size of food particles by contractile activity, and facilitates the movement of triturated chyme into the small intestine (Schulze, 2006). Gastric contractile activity can either be sustained for a period of time (tonic) or peak and subside (phasic) (Lentle and Janssen, 2011a). Tonic contractions, seen mainly in the fundus, allow for both the accommodation of meals and on-flow of digesta, which occurs without significant mixing. Phasic contractions of the antrum and pylorus contribute to the trituration of solid food particles readying them for expulsion into the duodenum (Lentle and Janssen, 2011b, Lentle and Janssen, 2011a). Antral contractions push gastric chyme towards the pylorus, while contraction of the gastric wall partially occludes the lumen, and forces a proportion of digesta to flow in a retrograde direction (Lentle et al., 2010). This retrograde fluid motion exerts shear forces onto particles of food facilitating their gradual erosion (Marciani et al., 2001a, Lentle et al., 2010, Lentle and Janssen, 2011b). The degree of gastro-mechanical mixing is determined by the physicochemical composition of chyme (Marciani et al., 2001b). The physical nature and nutrient composition of a meal can modulate gastric contractile activity (Lentle et al., 2010), flow of digesta (Lentle and Janssen, 2010), the admixture of digestive enzymes with digesta; and the rate of gastric emptying (Marciani et al., 2001b). Gastric emptying can take up to 3 hours depending on the composition of the meal (Marciani et al., 2001b, Collins et al., 1991).

1.1.3 Small intestine luminal digestion: biliopancreatic digestion

Chyme, triturated in the stomach, is discharged into the duodenum. Chyme empties from the stomach during both contractile and non-contractile events (Hausken et al., 2002), in a process mediated by changes in gastric tone (Lentle and Janssen, 2011b). There is a bidirectional flow of chyme across the pylorus that augments the admixture of gastric and duodenal contents; and may augment nutrient hydrolysis (Hausken et al., 2002). The speed at which the stomach empties is modulated by nutrient-mediated neurohumoral feedback, which occurs in a complex bioregulatory manner. For example, the enterokine cholecystokinin (CCK) not only regulates gastric secretion, motility, and emptying, it concomitantly stimulates the release of biliopancreatic secretions (Schneeman, 2002) and brush border (BB) enzymes, such as enteropeptidase (Götze et

al., 1972), in response to the nutrient composition of chyme (van Aken, 2010). Many pancreatic proteases are secreted into the duodenum as inactive zymogens that require activation by enteropeptidase; BB enteropeptidase activates pancreatic trypsinogen, forming trypsin, and trypsin in turn activates the other proteolytic zymogens (Stevens, 2000). Pancreatic secretions include pancreatic α-amylase, endopeptidases, carboxypeptidases, and lipolytic enzymes (Beck, 1973). During pancreatic exocrine secretion bile is released from the gallbladder, and when mixed with chyme, promotes the emulsification of lipids, augments lipolysis, and promotes the denaturation of proteins (Maldonado-Valderrama et al., 2011). Hence, the mixing of digestive secretions ensures optimum digestive efficiency.

Table 1. Local conditions in the digestive compartments

Compartment	pH	Secretions	Volume (day)	Residence time
Mouth	~6 (basal) ~8(stimulated)	α-amylase Lingual lipase Mucus Antibacterial compounds	1-1.5 litres	Seconds → minutes
Stomach	1-2 (fasting)* 2-5 (fed)*	HCl Pepsinogen Intrinsic factor Mucus Bicarbonate	1.5→2 litres	8-15 minutes (fasting)* 0.5-3 hours (fed)*
Duodenum	4-5.5*	*Brunners gland (pH 8-8.9)* Bicarbonate Mucin Epidermal growth factor *Liver →Bile (pH 7.8)* Bile salts Bicarbonate Organic waste *Pancreatic juice (pH 7.8)* Bicarbonate Digestive enzymes	0.2 litres 0.5-1 litres 1-1.5 litres	0.5-0.75 hours*
Jejunum	5.5-7*			1.5-2 hours*
Ileum	7-7.5*			5-7 hours*
Succus entericus (small intestine)	7.5-8	Mucus Brush border enzymes Enteropeptidase Oligosaccharidases Oligopeptidases Dipeptidases Nucleotides Nucleosides Lipases Cholesterol esterase	1-2 litres	

This table has been adapted from Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats (DeSesso and Jacobson, 2001) *(Oomen et al., 2002) $^{\gamma}$ trypsinogen, chymotrypsinogen, proelastase, procarboxypeptidases, lipase, pro-colipase, propphospholipaseA_2, cholesterol esterase, ribonuclease, deoxyribonuclease and others.

The type of contraction, and the degree of mixing induced in the small intestine, is dependent on local and neuronal stimuli (Lentle and Janssen, 2011e). Tonic and phasic contractions of either the circular or longitudinal muscle of the small intestine alone can displace digesta into adjacent segments of the digestive tract, and contribute to vorticeal mixing (Lentle and Janssen, 2011e), while coordinated contraction of small intestinal longitudinal and circular smooth muscle induces the flow of digesta (peristalsis) and facilitates mixing (Lentle and Janssen, 2011e). Mixing and flow during peristalsis is dependent on the amplitude of contraction (Lentle and Janssen, 2011e) and the physicochemical properties of digesta (Lentle et al., 2005). Viscous digesta imposes a constant smooth laminar flow with little mixing, whereas a low viscosity (watery) digesta allows a turbulent flow to develop that facilitates the mixing of digesta (Lentle and Janssen, 2011e). Although estimates of small intestinal transit can be as high as 7-10 hours (Oomen et al., 2002) most estimates suggest intestinal transit times of 3-6 hours (DeSesso and Jacobson, 2001, Maqbool et al., 2009, Rao et al., 2009).

1.1.4 Small intestinal luminal digestion: brush border (BB) digestion

In the small intestine, BB hydrolases, originally associated with the apical microvilli of the small intestine, act in concert with pancreatic enzymes to complete the process of digestion, prior to absorption. ‘Brush border’ is a term used to describe microvillus projections extending from the apical surface of the small intestinal enterocyte membrane. The BB microvillus is a dynamic organelle that sheds membrane vesicles into the peri-apical space (McConnell et al., 2009). These vesicles have catalytically active enzymes incorporated into their membrane (McConnell et al., 2009). The liberation of brush border membrane vesicles (BBMV) shifts the site of BB digestion into the lumen of the small intestine. Proteomic analysis suggests that approximately one sixth of the 646 proteins

associated BBMV are digestive hydrolases; these include oligosaccharidases, oligopeptidases and lipolytic enzymes (McConnell et al., 2011).

1.2 *In vitro* digestion

Although physiologically relevant, evaluation of the digestive processes by *in vivo* methods is expensive, can be difficult to control, and can be associated with ethical issues, necessitating the development of alternative methods for evaluating digestive efficiency. The pressure to develop innovative and physiologically relevant *in vitro* digestion models is driven by the need for an accurate and reproducible process that reduces the variation within and between human participants and completes the digestion of a particular food. However, the results of many *in vitro* digestions can be misleading if they fail to incorporate aspects of gastrointestinal physiology, such as cellular surface properties, motility, residence time, ongoing secretion, temperature, pH, food disintegration/erosion, rheology of digesta, gastric emptying, transit times, the admixture of food with digestive enzymes, and BB digestion. An introduction into *in vitro* methodology will now be given followed by a critique of current methods used for *in vitro* digestion.

1.2.1 Methods used for *in vitro* digestion

In vitro methods tend to recapitulate the sequential transit of digesta through the digestive tract. But many of these methods do not replicate continued secretion, the removal of digestive products and do not account for aspects such as hydration and shear. Local conditions can be altered to emulate buccal, gastric and small intestinal digestion depending on the scope of the study. A buccal phase often employs a blender, sieve or homogeniser to simulate the breakdown of food, but some methods also use human participants to chew food before it is added to an *in vitro* digestion (Woolnough et al., 2010).

Most *in vitro* digestion methods alter the pH and incubation time of the gastric and intestinal phases, and the addition of phase specific digestive enzymes further serves to differentiate digestive compartments. Surfactants and enzymes are often added, in un-physiological ratios, and physical processes are used to mimic gastric and small intestinal motility, by agitating digesta at forces not in keeping with those seen *in vivo*. In response to the need for standardisation of *in vitro* methods, and based on international consensus (COST Infogest network), a standardised static *in vitro* digestion method suitable for food has recently been published (Minekus et al., 2014). However, the method is acknowledged as static, and represents the enzymes and ionic conditions of luminal digestion. It does not yet include the mechanical, dynamic/termporal, or BB components of a complete and valid *in vitro* digestion system.

Physical processing is emulated in various ways and includes for instance, a stirring bar at 30 rpm (gastric digestion) (Kong and Singh, 2009); a shaking water bath at 250 rpm (small intestine) (Wright et al., 2008); and a shaking platform set to 12 oscillations per minute (small intestine) (Jin et al., 2006). A more advanced mechanical method, the Human Gastric Simulator (HGS), applies a force of 2.56 ± 0.45 N (lowest setting) to gastric contents (Kong and Singh, 2010) inducing shear rates far in excess of those measured *in vivo, i.e.* between 0.58 and 0.78 N (Marciani et al., 2001a).

More advanced models emulate the gradual transit of digesta and incorporate motile aspects that aim to mimic the physiological changes in the digestive tract. Most of these models are controlled by computers and allow for the continuous addition of digestive secretions. A few of the models currently in use will be described here. The TNO gastrointestinal tract model (TIM-1) consists of tubular compartments that sequentially emulate the gastrointestinal tract, and allow for the removal of dialysed effluent. TIM-1 includes a stomach and three small intestinal compartments (duodenum, jejunum, and

ileum) which consist of inner flexible vessels surrounded by glass jackets which have water running through them to maintain a constant temperature. Due to complexity TIM-1 is low throughput and expensive to operate per sample.

The water compartment can fluctuate in volume resulting in changes to the pressure exerted on the compartment within. Peristaltic valves connect digestive compartments and samples of dialysed effluent can be collected throughout the digestive phase (Blanquet et al., 2004). Although this method moves somewhat closer to the motility seen in the gut it does not mimic both circular and longitudinal muscle contractions and the various types of motility induced. The TIM-1 model can simulate the continued addition of digestive secretions by adding gastric juices at 0.5 ml/min and duodenal juices at 1 ml/min (Larsson et al., 1997). This method has also evolved to incorporate *Rhizopus* lipase, in the buccal and gastric phases, to simulate lingual lipase and gastric lipase respectively, and these authors also use pancreatic juice instead of individual enzymes during the duodenal phase (Verwei et al., 2003).

The Human gastric simulator (HGS) simulates the digestion of bulk foods in the stomach. The latex stomach has motorised rollers and belts that move along the gastric wall simulating peristaltic movement (the force can be altered), and allowing for a higher degree of force to be generated and applied at the bottom of the chamber, as seen in the antrum (Kong and Singh, 2010). The HGS is temperature controlled, has a mesh lining with a pore size of 1.5mm to simulate gastric sieving, and has a peristaltic pump which allows the operator to adjust the delivery of gastric juice.

The Dynamic Gastric model (DGM) is composed of a piston, and a body, which mimics the mechanical action and processing within the stomach. The fundus and antrum are represented by two chambers. The fundus undergoes pulsatile activity and mimics the mixing and diffusion profiles of acid and enzymes the antrum, and applies shear forces

promoting the physical breakdown of food particles (Wickham et al., 2009). Gastric secretions, similar in composition to *in vivo* secretions, are added and the DGM allows the temporal release of chyme.

The small intestinal model (SIM) consists of a porous inner membrane and an outer impermeable tube, with surge tanks to allow fluid movement, inflatable cuffs to produce segmentation like fluid movement, and a peristaltic pump to produce peristaltic movement (Tharakan et al., 2010). Mixed models of the described methods can also occur; for example TIM-1 and colon cancer 2 (CaCo-2) cells (Déat et al., 2009), the static model and CaCo-2 cells, (Glahn et al., 1998), and the DGM and static small intestinal model (Pitino et al., 2010).

Other additions to *in vitro* methods include flushing with nitrogen to minimise oxidative damage (Wright et al., 2008), and dialysis tubing (Macagno et al., 1982) or differentiated CaCo-2 cell lines to emulate absorption (Jin et al., 2006). Dialysis tubing will not emulate active transport, such as that mediated by proteins like the sodium-glucose linked transporter 1 (SGLT1); and being differentiated colon cancer cell lines, CaCo-2 cells only approximate small intestinal cell function, the surface area available for absorption in the CaCo-2 model does not come close to the 100,000 cm^2 seen *in vivo* (Ekmekcioglu, 2002).

1.3 Are current methods of *in vitro* analysis physiologically valid?

1.3.1 Variables identified that may improve the buccal phase of digestion

Mastication varies widely between individuals and is dependent on factors such as life stage, dentition (Mishellany et al., 2006, Pedersen et al., 2002), salivary flow (Pedersen

et al., 2002), and neuromuscular function (Van der Bilt et al., 2006). The type of food, number of chews, duration of chewing, frequency of chews, the size of food particles and the size of the swallowed bolus, varies between people and with degree of hunger (Hutchings et al., 2011). Hence, incorporating an homogenous slurry, with food particles of a uniform size, would not be in keeping with the heterogeneous boli seen during *in vivo* buccal processing. Components that may modulate the digestive process are discussed below.

Saliva

In circumstances where *in vitro* methodology simulates buccal digestion salivary secretions are usually artificially formulated (Kong and Singh, 2010). The individual components contributing to simulated saliva are sourced from a variety of suppliers. Pancreatic amylase is normally added to artificial saliva so will have different biochemical properties to salivary amylase, *i.e.* pH optima and stability (Stiefel and Keller, 1973). Likewise the addition of *Rhizopus* lipase to simulate lingual lipase (Verwei et al., 2003) will only approximate normal lingual lipase activity. Although both enzymes demonstrate similar substrate specificity, in their preference for medium chain fatty acids (Hiol et al., 2000, Liao et al., 1984), but lingual lipase is inhibited by bile (Liao et al., 1984) while *Rhizopus* lipase activity is augmented (Hiol et al., 2000).

The type of mucus used in artificial saliva is generally sourced from pig gastric mucin which differs in its biochemical and physical properties from buccal mucin. This is due to the fact that different organ systems produce different mucin types that have differing physicochemical properties (Bansil and Turner, 2006). The best option would be to use human participants to masticate food prior to *in vitro* digestion. During mastication food is bathed in saliva and agglomerated into a swallow safe bolus prior to swallowing; in the

stomach it can remain predominantly intact for a period of time depending on bolus viscosity (Marciani et al., 2001b). High pressure during oesophageal transit to the stomach may also compact the bolus allowing it to stay intact longer (Chen and Lolivret, 2011, Raut et al., 2001, Pal et al., 2003). This would give buccal enzymes time to digest nutrients prior dissolution by the mechanical gastric phase. This factor is not accounted for in current *in vitro* methods.

Salivary pH and enzyme activity

The pH of artificial saliva differs between resting and stimulated conditions, so should be accounted for when developing *in vitro* methods. Although saliva has a basal pH of approximately 6 the pH will rise to around 8 when stimulated (DeSesso and Jacobson, 2001). When artificial saliva is used, for *in vitro* digestion, the pH varies considerably, *e.g.* 5.8 (Abdelbary et al., 2005), 6.5 (Oomen et al., 2004) and 6.9 (Laurent et al., 2007). As *in vitro* digestion procedures simulate the post-prandial phase having a pH closer to 8 would be physiologically relevant. This is particularly apt as the activity of buccal enzymes is dependent on the pH of salivary secretions. Salivary amylase has a pH optimum of 5 - 7 (Stiefel and Keller, 1973, Rosenblum et al., 1988), but activity is progressively inhibited at a pH < 3 (Rosenblum et al., 1988). Lingual lipase is stable between pH 2.5 and 6 (Fink et al., 1984, Liao et al., 1984). The lower pH optimum for lingual lipase suggests that continued digestion in the gastric environment is likely to occur on the outside of the bolus and that amylase activity is more likely to continue within the confines of the bolus.

1.3.2 Variables identified that may improve the gastric phase of digestion

The gastric phase of digestion commences when a bolus of food arrives in the stomach. As mentioned earlier a food bolus can remain intact for a significant period of time, before digestive secretions are able to complete the erosion and dilution of the food bolus. Marciani et al (2001b) illustrated that a moderately viscous bolus of food can remain predominantly intact for more than an hour after ingestion. Current *in vitro* methodologies add a slurry of food particles into a gastric phase and immediately initiate physical processing, often at non physiological rates. This may not be the best approach for promoting ongoing buccal digestion in the artificial stomach.

Temperature

The temperature of food ingested can vary markedly, but once food is swallowed it must acclimate to body temperature prior to processing. Hence, the frequency of gastric myoelectrical events transiently decreases following the ingestion of a cold (4^{o}C) meal, and increases following the ingestion of a high temperature (55^{o}C) meal, compared to a meal of 37^{o}C (Verhagen et al., 1998). Further, gastric emptying is found to increase significantly in the early phase of digestion when the meal temperature is 60^{o}C compared to 37^{o}C (Mishima et al., 2009). This may result from an increase in myoelectrical activity (Verhagen et al., 1998). Hence, adding masticated food into an *in vitro* digestion at the temperature at which it is normally consumed more closely emulates the normal digestive processes. Although the temperature of the food is not taken into account in most *in vitro* digestion procedures they are all generally undertaken at a temperature of 37^{o}C, reflecting normal physiological conditions.

Gastric pH

The pH and secretory rate of digestion fluids differs between *in vitro* experiments, as it does between people. Most *in vitro* methods do not sequentially acidify the gastric phase, failing to reproduce the normal pH cycle, and as such do not account for temporal changes in acidity. Further, many do not account for the continuous secretion, absorption, admixture of gastric juice with digestive contents and on-flow that is seen *in vivo* during normal digestive processes (Lentle and Janssen, 2011c). The pH of gastric chyme rises from approximately 1.5 (fasting) to around pH 5 (post-prandial) (DeSesso and Jacobson, 2001, Oomen et al., 2002, Maqbool et al., 2009), but the continued secretion of acidic gastric juice will gradually acidify chyme to a pH of 1-2 (Ekmekcioglu, 2002). Without the sequential addition of acidic gastric juice, during *in vitro* digestion, the pH of chyme will continue to rise over the period of incubation (Chen et al., 2011). This decrease in acidity may in turn affect the activity of pepsin and gastric lipase. Pepsinogen requires a pH of less than 5 for activation (Richter et al., 1998). Gastric lipase requires a pH of 2 in order to undergo initial interfacial binding at the lipid/water interface, but maximal lipase activity occurs at a pH of 4 and 7.5, for the digestion of long chain triglycerides and vinyl butyrate respectively(Chahinian et al., 2006).

Composition of gastric juices

Another factor that should be addressed is the composition of gastric fluid. During the fasting state the osmolality of the stomach is approximately 191 ± 36 (114-230) mOsm/kg (Lindahl et al., 1997). The ionic composition of the major electrolytes in gastric secretion is: sodium 68 ± 29; chloride 102 ± 28mM; potassium 13.4 ± 3mM; and calcium 0.6 ± 0.2 mM (Lindahl et al., 1997). Electrolytes are not always incorporated into gastric or small intestinal digestions, but this omission could alter electrostatic forces acting on digesta.

For example, the ionic strength and composition of gastrointestinal contents can result in electrostatic interactions with dietary lipids that alter digestibility (McClements et al., 2008).

The structure of a lipid emulsion during digestion is important for the digestion of lipids as the surface area of a lipid droplet determines the area available for the absorption of lipase at the lipid/water interface (Golding et al., 2011). Emulsification occurs in response to mechanical agitation in the presence of endogenous and dietary amphiphiles (compounds that display both lipophilic and hydrophilic properties) (Golding and Wooster, 2011). Amphiphilic compounds partition to the oil water interface and stabilise the emulsion; without adequate emulsification the lipid phase will form a layer on top of digesta (Schulze, 2006), which will reduce the amount of surface area available for the lipase to act at the oil water interface. The degree of lipid emulsification will then determine the neurohumoral enterokine response which will subsequently modulate gastro-duodenal motor activity *in vivo, i.e.* an increase in the lipid droplet size in emulsions results in attenuation of the enteroendocrine response and gastrointestinal motility (Seimon et al., 2009). Conditions that do not emulate the normal process of lipid emulsification may therefore restrict the degree of lipid digestion.

Physical processing in the stomach

The obvious limitation seen in many *in vitro* models is a lack of physical processing (Wickham et al., 2009). Mechanical forces in addition to acid and enzymatic hydrolysis, facilitate the physical breakdown of food particles in the stomach by generating fluid shear, which results in the grinding and crushing of food particles (Ferrua et al., 2011). Experiments with simple models have shown that it is impossible to reach forces nearing antral forces seen *in vivo* (Kong and Singh, 2008). The frequency of myoelectrical activity

in the stomach is 3 cycles per minute (Ekmekcioglu, 2002), which produces forces between 0.58-0.78N (Marciani et al., 2001a), which induces fluid motion of approximately 7.5 mm/s (retropropulsive jets) that have a maximal shear force of 10-30 Pa (Pal et al., 2004). Basic *in vitro* digestion models use Teflon stirrers, paddles, magnetic bars or shaking platforms to mix digesta, but these will not come close to simulating the type of fluid motion or pressure forces that are exerted on food particles during normal digestion.

More advanced gastric models utilise flexible materials to emulate the stomach wall and a mechanical apparatus, to mimic tonic and phasic contractility, and mixing profiles. For example the body of the DGM stomach models the mass transfer of digestive secretions into food, as well as mixing and emptying into the antrum (Wickham et al., 2009). The antrum subjects food particles to shear forces that have been measured using Echo planar magnetic resonance imaging (EPMRI) and correlation studies (Wickham et al., 2009). Dynamic mixing such as this results in heterogeneous gastric mixing (Pitino et al., 2010) as seen *in vivo* (Marciani et al., 2001b). The HGS produces a ring of contraction that works down to a tapered end producing retropropulsive fluid motions (Kong and Singh, 2010). However, the amount of force produced by HGS is between 2.56 and 3.39 N (Kong and Singh, 2010), which is much higher than seen in normal digestion.

Another pattern of gastrointestinal motility occurs during the inter-digestive phase, but this is not incorporated into *in vitro* procedures. The migrating motor complex (MMC) is a series of myoelectrical excitation/contraction waves that propagate and spread distally from the stomach to the distal large intestine in order to drive remaining food particles and non-digestible detritus through the gastrointestinal tract (Huizinga and Lammers, 2009). The MMC occurs in four phases; rest (phase I), irregular contractions (phase II), regular rhythmic contractions (phase III) and a rapid decrease in activity (phase IV)

(Peeters et al., 1983). Non digestible solids (*i.e.* fibrous material and debris) empty predominantly during phase III of the migrating motor complex (Cassilly et al., 2008). The application of MMC to *in vitro* digestion is yet to be attempted.

Gastric residence time

Gastric emptying time is dependent on the composition and viscosity of meals. Echo planar magnetic resonance imaging has demonstrated that a low viscosity non-nutrient meal has a gastric emptying half-life ($T_{50\,\%}$) of 32 ± 7 minutes compared to 76 ± 6 minutes for a high viscosity nutrient meal (Marciani et al., 2001b). Most methods do not take the composition of individual meals into account when planning the timing of the gastric phase, but tend to have a standard incubation time regardless of nutrient or physical composition. This could alter the composition of digestive products, and should be considered.

1.3.3 Variables identified that may improve the duodenal (pancreatic) phase of digestion

As the small intestine is the primary site of digestion, having a complete understanding of the physiology is important. As previously mentioned, a bidirectional flux of chyme occurs between the stomach and duodenum during gastric emptying (Hausken et al., 2002), which may result in the augmentation of enzyme activity in one or both digestive compartments. Investigating this phenomenon may be worthwhile.

Temperature

The temperature at which an *in vitro* digest is conducted is very important as it will impact on the activities of enzymes. Since the core body temperature of people, assessed rectally,

averages approximately 37°C (Sund-Levander et al., 2002), conducting digestions at this temperature should achieve more realistic results. Temperature is also important when choosing an enzyme source, for a compartment of the digestive system, because enzymes vary in their tolerance to temperature. For example, the α-amylase family of enzymes are similar in structure and function, but their activity is specific to the site of action. Salivary amylase is significantly more stable at high (>45°C) temperatures compared with pancreatic α-amylase, which markedly loses activity at temperatures >45°C (Stiefel and Keller, 1973).

Enzyme activity and pH

The activities of enzymes can be altered by the presence of other enzymes and digestive secretions, during the digestive process. The enzymatic activity of pancreatic proteases are dependent on the degree of acid hydrolysis and proteolysis completed in the stomach. A higher than normal or unadjusted gastric pH will reduce the amount of protein that is degraded by acid hydrolysis and proteolysis, which reduces the number of peptides that are available for the action of pancreatic peptidases (Eriksen et al., 2010).

The pH of the intestinal milieu changes from the proximal to distal small intestine, see table 1. Such changes are important because the pH of particular intestinal segments provide an environment that allows enzyme activities to reach maximal velocity. The pH optimum for the enzymatic activity of pancreatic lipase enzymatic activity is approximately 7.5 (Vandermeers et al., 1974, Carrière et al., 2000). However, pancreatic lipase undergoes interfacial binding at the lipid/water interface at a lower pH between 4 and 6.5 (Ranaldi et al., 2008). This pH range is reminiscent of the pH of chyme soon after arrival in the proximal duodenum.

The activities of the enzymes chosen for an *in vitro* assay should approximate those seen *in vivo*. Many methods appear to randomly choose concentrations for pancreatic enzymes, or work on the principle that the concentration that works most efficiently is also the most physiologically relevant. For example pepsin 37.3 U/ml, pancreatic amylase 27 U/ml, and bile acids 2.7 mM have all been measured *in vivo* (Ulleberg et al., 2011). The concentrations of the secretory products used for *in vitro* digestions, are used in excess of those seen *in vivo, i.e.* 1100 U/ml pepsinogen (Larsson et al., 1997), 300 U/ml pancreatic amylase (Pitino et al., 2010) and 12 mM mixed bile salts (Golding et al., 2011). The digestion itself may run more efficiently, but results will not be indicative of what happens *in vivo,* as the increased activity may have unforeseen effects such as inhibiting other enzymes.

Ionic composition of luminal contents

During the fasting state the osmolality of the jejunum is 271±15 (218-292) mOsm/kg which is higher than in the stomach (Lindahl et al., 1997). The ionic composition of major electrolytes are: sodium 142 ± 13 mM; chloride 126 ± 19mM; potassium 5.4 ± 2.1; and calcium 0.5 ±0.3mM (Lindahl et al., 1997). Ionic composition is generally not adjusted when an *in vitro* digestion transits from the gastric phase into the intestinal phase, but the composition of individual electrolytes is important. Pancreatic enzymes need the presence of electrolytes such as calcium ions in order to function normally; in the case of pancreatic amylase the presence of calcium is essential for function as calcium determines enzymatic activity by stabilising the active site, and determining the tertiary structure (Buisson et al., 1987).

Enzyme interactions

The activity of many pancreatic enzymes are altered by the presence of other enzymes and/or digestive secretions. When a researcher chooses to use only one or two enzymes in an *in vitro* digestion they may be restricting the number of digestion products, as enzymes are known to act synergistically during the digestive process (Savoie and Charbonneau, 1990). For example, in a milk emulsion the digestion of lipids is blocked by the partitioning of proteins across the oil-water interface; proteolytic enzymes hydrolyse these proteins resulting in the augmentation of lipolysis as peptides fragments diffuse away (Lueamsaisuk et al., 2013). The complete digestion of lipids requires the concerted action gastric lipase followed by pancreatic lipase, but the function of pancreatic lipase is dependent on the presence of the coenzyme co-lipase (Carrière et al., 2000, Bernbäck et al., 1990). Co-lipase promotes lipolysis by binding to the C-domain and the lid which structurally stabilises lipase (van Tilbeurgh et al., 1993). Amphiphiles, such as bile acids, are required to emulsify lipids and to reduce surface tension at the lipid/water interface in turn facilitating the binding of lipase to co-lipase (Chahinian et al., 2006). Many *in vitro* methods do not include co-lipase in the reaction mixtures (Mun et al., 2007, Sandra et al., 2008, McDougall et al., 2009), but most tend to use bile acids to improve lipolysis. However, bile acids are generally omitted in reaction mixtures that do not specifically measure lipolysis. It has been shown that bile acids augment the activity of other enzymes. The activity of trypsin and α-chymotrypsin activity are enhanced by the presence of bile acids *in vitro* because bile destabilises the tertiary structure of proteins (Gass et al., 2007). Using a crude mixture of biliopancreatic secretions containing a physiological complement of digestive enzymes may lead to more bio-relevant derivatives being found during the digestive process. As previously mentioned the brush border enzyme enteropeptidase is required to activate trypsinogen,

which in turn activates the other pancreatic zymogens (Light and Janska, 1989) so not adding enteropeptidase or omitting the BB phase may be detrimental to peptidolysis.

Motility and residence time

The frequency of peristaltic events in the duodenum, and jejunum and ileum is 11.7 and 8.9-9.8 cycles per minute respectively (Ekmekcioglu, 2002). Unfortunately most experiments do not measure/describe the force of "contractions" in the *in vitro* small intestinal segment so it is difficult to tell whether they approximate physiological conditions. The types of small intestinal contraction elicited *in vivo* cannot be mimicked *in vitro* as they require the co-ordinated contraction of both circular and longitudinal muscle. At this stage this has not been accomplished. Therefore, the contractile force transferred to digesta in an *in vitro* vessel is never going to be equivalent to that seen *in vivo* and is unlikely to induce the same flow patterns.

The timing of the *in vitro* intestinal phase varies, but generally runs between 1.5-2 hours. Transit through the duodenum, jejunum and ileum is 0.5-0.75, 1.5-2, and 5-7 hours respectively (Oomen et al., 2002). Therefore, a two hour digestion time does not represent normal intestinal residence. Adjusting the time line may result in a more efficient digestion.

1.3.4 Brush border digestion

A survey of the literature has shown that incorporating a suite of BB enzymes into an *in vitro* digestive phase for the digestion of polyphenolics is yet to be achieved, although BB digestive phases have been used for the digestion of proteins (Picariello et al., 2010). However, the rationale and validation of its use appear to be lacking (Petrilli et al., 1984, Shan et al., 2002, Mamone et al., 2015). Other systems partially utilise BB hydrolytic

capabilities, for example, amyloglucosidase has been added to some *in vitro* digestion systems to allow for the complete depolymerisation of carbohydrates (Mishra et al., 2008).

The significance of BBMV enzymes is evident when some of these digestive enzymes are absent or malfunction *in vivo*. Oligosaccharidase deficiency can lead to osmotic diarrhoea and digestive malabsorption (Bayless and Christopher, 1969), and enteropeptidase deficiency manifests with protein malabsorption, diarrhoea, oedema and failure to thrive (Holzinger et al., 2002). Logically developing an effective brush border digestive phase is imperative to understanding terminal small intestinal digestion; particularly with the current interest in functional foods and bioactive compounds. Plant polyphenols for example are de-glycosylated by the BB enzyme lactase-phorizin hydrolase (LPH) (Sesink et al., 2003, Day et al., 2000), which may result in the strengthening or attenuation of their antioxidant or nutraceutical activities.

1.3.5 Accounting for cellular surface properties and absorption

Dialysis membranes

Some *in vitro* procedures involve a two compartment model that utilises dialysis membranes to mimic absorption. In the two chamber system the composition of the fluid in each compartment and the molecular cut off for the dialysing membrane should be matched to what is physiologically normal (Ekmekcioglu, 2002). This is very difficult to accomplish. The molecular cut off point for dialysis filters used for *in vitro* studies varies between projects. Dialysis membranes with molecular cut-offs between 5,000 – 10,000 Da are often used (Tharakan et al., 2010, Van de Wiele et al., 2007). However, we know from permeability experiments that the permeability of a membrane is not determined solely by the molecular size or the diameter of molecules (Artursson et al., 1993). Factors

including the surface properties of the molecules will determine diffusion or transport (Pappenheimer et al., 1997), which during *in vivo* digestion and absorption is coupled to, and modulated by, the hydrolysis and flux of nutrients from the intestinal lumen to the epithelial space and into the cell, via transcellular and/or paracellular mechanisms (Pappenheimer, 1993). Transporter mechanisms may involve precise molecular recognition and fit that cannot be replicated by the array of pores in a dialysis membrane. If a BB phase of digestion is not incorporated into an *in vitro* digest the size of the metabolites, obtained during digestion, may not be representative of what is actually absorbed across the enterocyte. Rather they might be metabolites that would be available for further digestion by BB enzymes.

Colon cancer cell lines

Colon Cancer 2 immortal cell lines are often used in absorption studies (Déat et al., 2009, Glahn et al., 1998). These cells are able to differentiate into cells resembling polarised small intestinal cells, and possess microvilli and digestive hydrolases. Functional features such as permeability, digestive enzyme activity and regulation of transport are determined by the number of passages and the type of culture used (Sambuy et al., 2005).

Care should be taken in extrapolating transport data from both dialysis membranes and CaCo-2 cells as factors such as fluid load and absorptive area differs from that seen *in vivo* (Ekmekcioglu, 2002). For example, the active transport of glucose via SGLT and the GLUT 1-4 glucose transporters is driven by the flux of glucose into the cell and its subsequent transfer into systemic circulation. Since CaCo-2 cell lines do not provide interstitial space or systemic circulation the flux of metabolites into and out of the cell does not occur and will not give realistic results.

Mucin

Mucin has been used in *in vitro* digestion procedures with the aim of acting as a diffusive barrier to replace dialysis tubing (Jin et al., 2006). However, the mucin used by these authors is dried porcine gastric mucin so its structure and functional properties are likely to be different to the mucin of the organ of interest. The type of mucin produced differs depending on the site of production in the digestive tract. There are at least 19 mucin genes known at present with different tissues expressing different combinations (Bansil and Turner, 2006), and all possess different functional properties (Toribara et al., 1997), such as gelation and aggregation (Bansil and Turner, 2006). MUC6 and MUC5AC mucins predominate in the stomach (Ho et al., 2004) whilst MUC2 predominates in the small intestine (van Aken, 2010). MUC6 is a disulphide bonded multimer with a high degree of resistance to proteolysis whereas MUC2 is less glycosylated and is more susceptible to the action of proteases (Toribara et al., 1997).

Most *in vitro* methods that incorporate mucin use a commercially available dried mucin, which is generally porcine gastric mucin. Dried mucin is difficult to dissolve and the structure and functional properties of a re-suspended mucus are altered with changes in pH and ionic strength (Bansil and Turner, 2006, Bhaskar et al., 1991). Further, the buffering capacity of re-suspended mucus is different to that of from native mucus (Schreiber and Scheid, 1997).

1.4 Variables identified that may improve small intestinal luminal digestion: Brush border digestion

There are many aspects of *in vitro* digestion that could be improved, but the principal issue is the absence of a functional BB phase of digestion. This is particularly evident when the terminal digestion of a food is of interest as most *in vitro* digestion

methodologies do not utilise the full complement of small intestinal digestive enzymes. As such, nutrients such as lactose, sucrose, maltose, peptides (for allergy studies), sphingolipids, and non-nutrient dietary components such as polyphenolic glycosides (thought to provide health benefits) are not hydrolysed. A BB digestive phase could allow researchers to assess the endpoint products of luminal digestion, and gain better understanding of the digestive products.

1.4.1 Developing a physiologically relevant BB *in vitro* model.

This thesis will use the digestion of model substrates to determine the suitability of the BBMV enzyme consortia for use as an adjunct to an *in vitro* digestive phase representing luminal digestion. If the method is applicable for use then the system will be tested in the presence of physiological concentrations of biliopancreatic secretions and will be validated using polyphenolic compounds as substrates.

This thesis was designed as a standalone project, but was part of a larger Ministry of business, innovation and employment (MBIE) project called Foods for Appetite Control (FFAC). The objectives the FFAC program were to demonstrate the effect of plant compounds on appetite control mechanisms, identify plant sources with required levels of active compounds, and to deliver foods with validated appetite control effects using a multifactorial approach to activate satiety.

1. **Short term satiety:** using bitter compounds to induce an enteroendocrine response to slow eating.
2. **Medium term satiety:** enhancing the delivery of carbohydrates to the ileum to trigger the ileal break.
3. **Long term satiety (next meal):** using dietary components to activate the colonic break.

This thesis aimed to better align *in vitro* digestion with *in vivo* digestion in order to provide a method for identifying foods containing polyphenolic compounds that triggered enteroendocrine responses associated with short term satiety. A short overview of this theory is detailed below

1.4.1.1 The digestion and metabolism of polyphenolics compounds at the brush border

Polyphenolics have recently been recognised for their potential as antioxidants (Liu, 2004, RiceEvans et al., 1996), and for their possible role in inducing satiety via the activation of bitter taste receptors in the gastrointestinal tract (Roland et al., 2011, Rozengurt, 2006, Jang et al., 2007). Evidence suggests that polyphenolic aglycones are comparatively better agonists of bitter taste receptors than the glycosylated forms (Roland et al., 2011). However, polyphenolic compounds are primarily found conjugated to carbohydrates in their natural state (Herrmann, 1976, Bravo, 1998). So it is important to investigate the digestion and derivatisation of these compounds by endogenous enzymes in the small intestine to determine what polyphenolics would be present to interact with bitter taste receptors of the adjacent mucosa. A BB *in vitro* system may have an important role in determining the fate/transformation of polyphenolics in the gut as D-glycosides are attached to polyphenolic compounds in the β-conformation (Herrmann, 1976), and so are hydrolysable by LPH, a β-glycosidase of the small intestinal BB (Nemeth and Piskula, 2007). A review of the digestion and metabolism of polyphenolic compounds will be incorporated into the introduction of chapter 8. Since current methods of *in vitro* digestion do not utilise a consortium of BB enzymes, the development and inclusion of a functional consortia of BB enzymes for testing of polyphenolic digestion may increase our understanding of this process. The effects of the digestion and metabolism of

polyphenolic compounds within the small intestine may have implications for the development of foods to increase satiety.

1.5 Aims, objectives and hypotheses

The aims of this thesis were threefold; firstly, to identify points within the small intestine where *in vitro* methods could be better aligned with the known *in vivo* function. Secondly, to develop and validate an enzyme consortium that would emulate intestinal brush border digestion in an *in vitro* system. Thirdly, to assess the deglycosylation of polyphenolic compounds using the BB *in vitro* methods.

The aforementioned aims were approached in the following manner experimentally:

- The secretion and action of BB digestive enzymes were investigated in order to define a method to isolate and purify a consortium of BB enzymes.
- The main BB enzymes involved in the hydrolysis carbohydrate and peptide oligomers were characterised.
- Testing to validate that BB enzymes function during *in vitro* digestion under conditions approximating those found in the small intestine.
- Testing to determine whether polyphenolic compounds are hydrolysed in a BB *in vitro* model.

In order to test the aims and objectives the following hypotheses will be tested experimentally. The overall hypothesis of this research thesis is that a rat BBMV preparation can be used *in vitro* to simulate BB digestion of polyphenolic compounds.

This can be further divided into these specific hypotheses

1. The kinetic activities of BB enzymes from different preparations of rat BBMV are not replicable

2. The activities of rat BB enzymes will be significantly inhibited by the presence of bile and/or pancreatic enzymes.
3. Rat BBMV-bound enzymes will have significantly different enzyme kinetics to those that have been solubilised from the BBMV by bile or pancreatin.
4. The kinetic activities of solubilised rat BB enzymes will vary significantly compared with BBMV-bound enzymes due to changes in pH.

1.5.1 Experimental work

Prior to the experiments proper, pilot studies were conducted to develop methods for the purification of the BBMV preparation, assays for measuring BB enzyme activities, and assays for testing the stability of digestive enzymes in BBMV preparations. The results of these method development experiments are presented in appendix 4. Digestive enzymes in the BBMV preparation were assayed in physiological concentrations and volumes of biliopancreatic secretions previously described in published literature (Chapter 4). A method for a BB *in vitro* digestive phase was then developed, and validated using polyphenolic compounds as substrates. The flow of experimental work is outlined in figure 1.

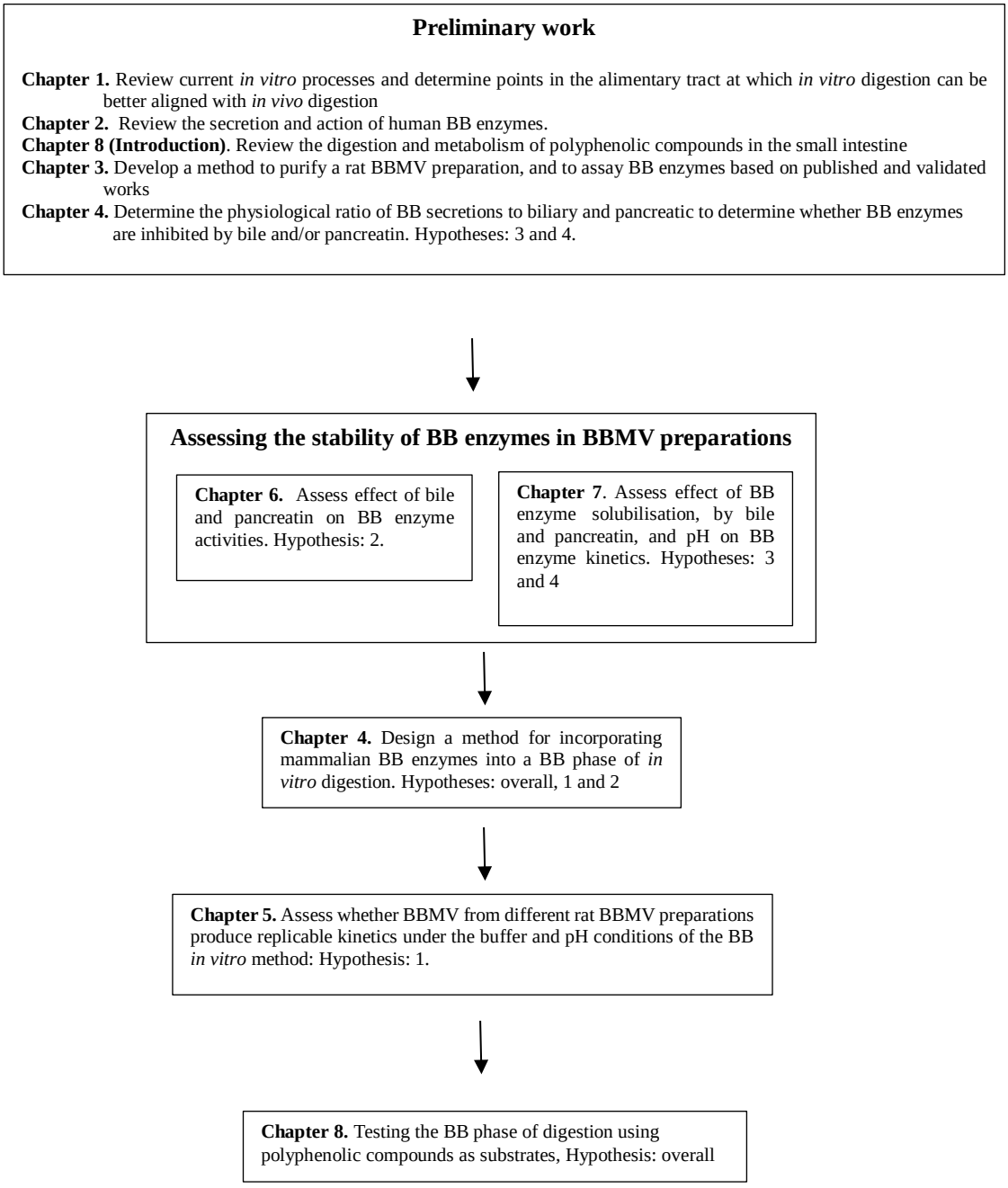

Figure 1. Schematic outlining the development of the *in vitro* BB digestion mode

1.6 Conclusion

Although *in vitro* methodologies for small intestinal digestion have progressed since its inception there are many aspects of digestion that are not addressed in current models. In developing an *in vitro* digestion method appropriate for determining the digestive fate of glycosylated polyphenolics there is the potential to apply the BB digestive phase as an adjunct to current *in vitro* models.

2 [1]Chapter 2. Literature review: The secretion and action of brush border enzymes in the mammalian small intestine

The previous chapter identified the small intestinal brush border (BB) as an important component to mammalian digestion that is not comprehensively addressed by current methods of *in vitro* digestion. In order to identify how to incorporate BB enzymes into an *in vitro* digestion it is important to understand the secretion and action of BB enzymes. Accordingly, this chapter will review the current literature pertaining to the small intestinal BB, and use that knowledge to guide the development of an adjunct BB phase. Microvilli are conventionally regarded as an extension of the small intestinal absorptive surface, but they are also, as latterly discovered, a launching pad for brush border digestive enzymes. Recent work has demonstrated that motor elements of the microvillus cytoskeleton operate to displace the apical membrane towards the apex of the microvillus, where it vesiculates and is shed into the peri-apical space. Catalytically active brush border digestive enzymes remain incorporated within the membranes of these vesicles, which shifts the site of BB digestion from the surface of the enterocyte to the peri-apical space. This process enables nutrient hydrolysis to occur adjacent to the membrane in a pre-absorptive step. The characterisation of BB digestive enzymes is influenced by the way in which these enzymes are anchored to the apical membranes of microvilli, their subsequent shedding in membrane vesicles and their differing susceptibilities to cleavage from the component membranes. In addition, the presence of active intracellular components of these enzymes complicates their quantitative assay and the elucidation of

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their dynamics. This review summarizes the ontogeny and regulation of BB digestive enzymes and what is known of their kinetics and their action in the peripheral and axial regions of the small intestinal lumen.

Digestive enzymes of the small intestinal brush border (BB) are responsible for the final stage of luminal digestion prior to absorption (Van Beers et al., 1995a, Semenza, 1986, Holmes and Lobley, 1989). These enzymes include a suite of oligopeptidases (*e.g.* aminopeptidases, carboxypeptidases, endopeptidases and dipeptidases), lipases (*e.g.* sphingolipid hydrolyzing enzymes and phospholipases) and oligosaccharidases (*e.g.* α1,4-glucosidases, α1,6-glucosidases, α1,β2-glycosidase, β1,4-glycosidases and α1,α1-glucosidase). Together these enzymes hydrolyse those nutrient oligomers that remain following buccal, gastric and pancreatic digestion. It is evident in their absence that BB enzymes have an important role in human digestion. A congenital deficiency of BB enteropeptidase (EC 3.4.21.9) results in gross protein malabsorption, nutrient deprivation, failure to thrive, diarrhoea and oedema (Holzinger et al., 2002, Tsai and Duggan, 2005). Likewise, congenital or acquired deficiency of BB lactase-phlorizin hydrolase (LPH, EC 3.2.1.108/3.2.1.62), congenital deficiency of sucrase-isomaltase (SI, EC 3.2.1.10/3.2.1.48), and deficiency in glucoamylase (EC 3.2.1.3) or trehalase (EC 3.2.1.28) all result in osmotic diarrhea, abdominal discomfort and flatulence (Tsai and Duggan, 2005).

The action of BB enzymes succeeds that of buccal, gastric and pancreatic digestion, and reduces nutrient oligomers in chyme to their component monomers. The gross digestion of nutrients occurs in the stomach by acidic (*i.e.* hydrochloric acid) and enzymatic hydrolysis (*e.g.* gastric lipase (EC 3.1.1.3) and pepsin (EC 3.1.23.1)). Pancreatic enzymes hydrolyze nutrient polymers remaining following prior digestion. These enzymes include endopeptidases (*e.g.* trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC

3.4.21.36)), carboxypeptidases (*e.g.* carboxypeptidase A (CPA, EC 3.4.17.1) and carboxypeptidase B (CPB, EC 3.4.17.2)), lipases (*e.g.* pancreatic triacylglycerol lipase (PTC, EC 3.1.1.3) and pancreatic bile salt activated lipase (BSAL, EC 3.1.1.13/3.1.1.3), phospholipases (*e.g.* phospholipase A2 (PLA2, EC 3.1.1.4)) and pancreatic α-amylase (EC 3.2.1.1.) (Beck, 1973). The resulting chyme is composed of short peptide oligomers and amino acids; branched carbohydrate oligomers, short glucose oligomers and α-dextrin; and lipids found in mixed micelles composed of free fatty acids and monoglycerides (Lowe, 2002, Beck, 1973, Bauer et al., 2005). Brush border enzymes may act on nutrient oligomers, whilst they are attached to brush border membrane vesicles (BBMV), after liberation from BBMV (by pancreatic proteolysis or BBMV membrane disruption) or following their liberation from the interior of enterocytes by lysis (Stevens, 2006, Semenza, 1986, Tso and Crissinger, 2006). In general BB enzymes are required to reduce nutrient dimers and oligomers into their component monomers before they can be absorbed via passive diffusion, endocytosis (Tso and Crissinger, 2006), transcellular carrier mediated transport (Tso and Crissinger, 2006, Daniel, 2004), or via solvent drag through intracellular junctions (Pappenheimer and Reiss, 1987). Some small di and tri peptides are transported directly across the apical membrane via peptide transporters such as the H^+ - peptide co-transporter PEPT1 (Daniel, 2004). Others may be absorbed via paracellular mechanisms, as is the in the case with some small hydrophilic peptides $\leq$ 2,000 Da (Pappenheimer, 1993). Peptides may undergo intracellular digestion by cytosolic enzymes, such as aminotripeptidase (EC 3.4.11.4) and Xaa-Pro-dipeptidase (EC 3.4.13.9) (Amidon and Lee, 1994). Others may undergo intracellular degradation by lysosomal and microsomal enzymes as do absorbed toxins and intracellular waste (Galand and Forstner, 1974b). Indigestible peptides may be excreted in urine or bile (Pappenheimer et al., 1997).

Early work showed that BB enzymes were intimately associated with the enterocyte BB membrane (Doell et al., 1965, Bourne and MacKinnon, 1943, Nachlas et al., 1960, Borgstrom et al., 1957) and it was hypothesized that this association facilitated the direct transit of products of enzymatic digestion to the interior of the enterocyte (Pappenheimer, 1993). More recently it has become evident that a proportion of these enzymes maintain a structural linkage with elements of the apical membrane and are actively budded off as BBMV into the adjacent peri-apical space (McConnell and Tyska, 2007, McConnell et al., 2009). Brush border enzymes may subsequently transit to all parts of the lumen in this form (McConnell et al., 2009, Halbhuber et al., 1994) or be released from BBMV by the action of biliopancreatic secretions (Auricchio et al., 1963, Semenza, 1986, Maestracci, 1976, Young and Das, 1990).

The dissemination of BBMV-bound and solublised BB enzymes throughout the small intestine is poorly understood. Although BBMV are recovered from intestinal contents (McConnell et al., 2009) the proportions of BBMV and solubilised BB enzymes that remain in the peri-apical space, or diffuse into the lumen, is not currently known. Hence, the primary site of digestion by BB enzymes is not known. Nevertheless, the admixture of pancreatic and BB enzymes are important for the normal process of digestion. Further, BB enteropeptidase is necessary for the activation of luminal pancreatic peptidases (Light and Janska, 1989), and peptidases secreted by the pancreas are required for the post-translational processing of some BB enzymes (Hauri et al., 1979). It is likely that the mucus layer overlying the epithelia retards the egress of BBMV from the peri-apical space into the lumen proper so that a significant proportion of BBMV would remain in close proximity to the intestinal mucosa to augment local nutrient hydrolysis, causing the concentration of nutrient products, and their lumen to enterocyte diffusion gradient, to be increased (Pohl et al., 1998).

The role of these enzymes in nutrient digestion has recently come under increased scrutiny; firstly, to gain a greater understanding of their distinctive dynamics, and secondly, from the need to incorporate such digestive processes into various *ex vivo* systems developed by the food and pharmacological industries (Liu et al., 2011, Sakuma et al., 2009, van der Burg-Koorevaar et al., 2011). This review details current knowledge regarding the genesis, action and dynamics of BB enzymes and their contribution to the digestive process within the mammalian small intestine.

2.1 Synthesis and secretion

2.1.1 Synthesis of BB enzymes

Brush border enzymes generally consist of two or more sub-units (Holmes and Lobley, 1989). Like other secretory proteins they are synthesized, either as single pro-enzyme polypeptide chains (Hauri et al., 1979), or as monomers (Kenny and Maroux, 1982, Danielsen et al., 1982), on ribosomes that coat the surface of the rough endoplasmic reticulum (RER) (Holmes and Lobley, 1989). The nascent proteins are subsequently translocated across (Lingappa, 1989, Blobel, 1980) and inserted into the RER membrane (Danielsen, 1982, Danielsen et al., 1983). The bulk of BB enzymes undergo co-translational and post-translational modification (Hurtley and Helenius, 1989) within the endoplasmic reticulum (Danielsen, 1992, Snider and Robbins, 1982), usually by site-specific mannose *N*-glycosylation (Danielsen, 1992) and dimerisation (Danielsen, 1994). Brush border enzymes are subsequently transferred to the Golgi apparatus where they may undergo further post-translational modification by site-specific proteolysis (Naim et al., 1987, Danielsen, 1990) and *O*-glycosylation (Rothman and Orci, 1992, Danielsen et al., 1984, Danielsen, 1990). There is some debate as to whether LPH undergoes

dimerisation in the endoplasmic reticulum (Grünberg and Sterchi, 1995) or within the Golgi apparatus (Danielsen, 1990).

Post-translational glycosylation is a feature common to BB digestive enzymes (The UniProt Consortium, 2014) and is thought to confer some protection from proteolytic degradation (Mer et al., 1996, Kingsley et al., 1986, Vaňková et al., 1994). The high degrees of glycosylation displayed by BB enzymes (Naim et al., 1988b, Naim et al., 1988a, The UniProt Consortium, 2014) are reflected in their greater molecular weights compared to similar enzymes from other sources (Holmes and Lobley, 1989). Thirty to forty percent of the molecular weight of human maltase-glucoamylase (MGAM, EC 3.2.1.20/3.2.1.3) (335 kDa) (Naim et al., 1988b) consists of carbohydrate (Naim et al., 1988b, Kelly and Alpers, 1973). Similarly, a quarter of the molecular weight of human SI (245 kDa) is attributed to glycosidic residues (Naim et al., 1988a). The corresponding pancreatic enzymes are not as extensively glycosylated and hence their molecular weights are much lower. For example human pancreatic α-amylase (EC 3.2.1.1) is not glycosylated (The UniProt Consortium, 2014, Zakowski and Bruns, 1985) and has a molecular weight of 54 kDa (Stiefel and Keller, 1973).

Brush border enzymes are preferentially transported to the apical membrane bound to an intracellular membrane (Danielsen et al., 1984, Bennett et al., 1974) via the network of microtubules (Danielsen, 1995, Hirschberg et al., 1998). It is not known whether the process by which these transported vesicles coalesce with, and become incorporated into, the apical membrane of the enterocyte is driven by differences in their thermodynamics (*i.e.* their surface energies) or by some other physiological process. A number of BB enzymes may undergo further post-translational processing within the lumen by pancreatic proteases (Sjöström et al., 1980, Sjöström et al., 1978, Hauri et al., 1979, Zecca et al., 1998). Alternately, isoforms of some BB enzymes may remain within the cell in a

soluble form (Seetharam et al., 1977) and others may be liberated directly into the peri-apical lumen (Götze et al., 1972). It is not clear whether these liberated enzymes are initially bound to BBMV, are secreted directly in a soluble form (Götze et al., 1972) or are a product of normal cell turnover, *i.e.* intracellular enzymes that are liberated from enterocytes that have been shed into luminal contents.

2.1.2 The anchoring of BB enzymes to the membrane

Brush border enzymes are generally anchored on the apical membrane of the enterocyte or BBMV with their catalytic sites projecting extracellularly into the intestinal lumen. This anchoring region is usually either a hydrophobic transmembrane peptide sequence, located close to one terminus so that the *C* or *N*-terminus is positioned within the cytoplasm (Semenza, 1986), or a covalently linked glycophosphatidylinositol (GPI) moiety (Low, 1989). The immobilisation of arrays of BB enzymes by attachment to BBMV is likely to facilitate their digestion of mutual substrates. Anchoring may also slow the diffusion of BBMV into the lumen allowing for their accumulation in the peri-apical space, which may allow for the pre-digestion of nutrients prior to reaching the enterocyte. Further, the attachment of enzymes to BBMV may ensure correct orientation (Killian and von Heijne, 2000), enzyme stability (White and Wimley, 1998) and activation (Sandermann, 1982, Wojtczak and NaŁĘCz, 1979) while proximity to the membrane may provide a solubilising surface for hydrophobic substrates (Zhou and Schulten, 1996). On the other hand soluble BB enzymes may diffuse more readily throughout the small intestine and be sterically less unencumbered by the proximity of other proteins. The relevant proportions of BBMV-bound or solubilised BB enzymes is not currently known. Further, it is not clear whether the liberation of BB enzymes from BBMV, by biliopancreatic secretions, improves the rate or extent of digestion.

Nascent pro-enzymes possess a distinctive signal peptide of hydrophobic amino acids at the carboxyl terminus that directs pro-enzyme to a putative endoplasmic reticulum transamidase that cleaves the signal peptide from the *C*-terminal and adds a pre-assembled GPI moiety (Low, 1989, Semenza, 1986, Gerber et al., 1992). In the case of alkaline phosphatase (ALP, EC 3.1.3.1), a 29 amino peptide sequence is removed from the carboxyl side of amino acid 179 and a pre-formed GPI anchor is then attached (Gerber et al., 1992). Other BB enzymes with GPI anchors include trehalase (Low, 1989, Ruf et al., 1990) and dipeptidase 1 (DP1, EC 3.4.13.19) (The UniProt Consortium, 2014, Hooper et al., 1990).

Hydrophobic peptide sequences are the more common method of anchoring BB enzymes. Most have a single pass peptide anchor that orientates across the hydrophobic portion of the apical membrane with the *N*-terminus located on the cytosolic side. This is known as a type II anchor. Brush border enzymes that possess type II peptide anchors include SI, MGAM, aminopeptidases (Semenza, 1986, Van Beers et al., 1995a), dipeptidylpeptidase IV (DPPIV, EC 3.4.14.5) (Benajiba and Maroux, 1980), and neprilysin (NEP, EC 3.4.24.11) (The UniProt Consortium, 2014).

Brush border enzymes with a type I peptide anchor have a single pass hydrophobic transmembrane sequence near the *C*-terminal, which anchors the enzyme to the membrane with the N-terminus situated in the lumen. Examples of enzymes with type I peptide anchors include LPH (Mantei et al., 1988), phospholipase B1 (PLB1, EC 3.1.1.4/3.1.1.5) (The UniProt Consortium, 2014, Takemori et al., 1998) and angiotensin converting enzyme (ACE, EC 3.4.2.-, 3.4.15.1) (Coates, 2003).

Brush border enzymes with peptide anchors of either kind can differ in their tertiary structures (*i.e.* be monomeric or dimeric) (Kenny and Maroux, 1982, Naim, 1993). A number of aminopeptidases, such as human aminopeptidase N (APN, EC 3.4.11.2)

consist of two homogenous proteins, each of which anchors independently to the membrane, whilst others (such as SI) consist of heterologous subunits that are anchored by a single sequence (Feracci and Maroux, 1980, Kenny and Maroux, 1982).

2.1.3 The formation and movement of BB membrane vesicles

Brush border membrane vesicles are formed at the apex of BB microvilli where the plasma membrane, including integral membrane proteins, becomes vesiculated and is shed into the peri-apical space (McConnell et al., 2009), see figure 2 and 3. The core of each microvillus comprises a longitudinally oriented array of 20–30 polarized actin filaments, each 50–60 nm in diameter, that are each linked to the plasma membrane by a series of myosin-1A cross-bridges (McConnell and Tyska, 2007). These are supported by an assortment of binding proteins that include villin (Bretscher and Weber, 1979), fimbrin (Bretscher and Weber, 1980), calmodulin (Howe et al., 1980) and esprin (Bartles et al., 1998) that extend into the terminal web of the enterocyte. Myosin-1A, the primary motor protein in this array, generates a shearing force between the plasma membrane and the actin fibers that is directed along the central axis of the microvillus. This force causes the plasma membrane to be progressively displaced towards the apex of the microvillus (McConnell and Tyska, 2007, McConnell et al., 2009). The process culminates in the budding of successive BBMV, each approximately 100 nm in diameter, from the tip of the structure (McConnell and Tyska, 2007). Hence, the location of BB enzymes shifts from the apical plasma membrane of the enterocyte to the plasma membrane of a BBMV in the peri-apical lumen (McConnell and Tyska, 2007, McConnell et al., 2009, McConnell et al., 2011, Halbhuber et al., 1994).

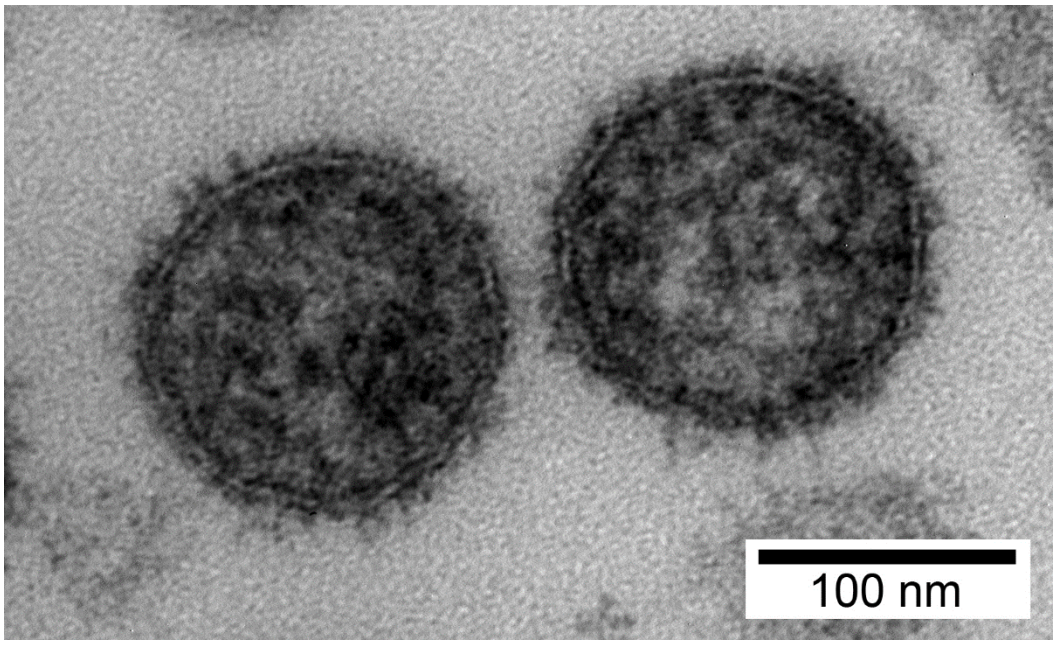

Figure 2. Transmission electron micrograph of BBMV

Crucial to vesicle formation is the final budding at the apex of the microvillus. It is possible that the energy generated in this process is able to establish sufficient local curvature of the membrane to cause droplet formation. However, it seems likely that the budding process is accompanied by selective re-absorption or incorporation of fatty elements at particular points in the apical plasma membranes (Brasitus and Dudeja, 1985) so as to reduce the local surface energy of the membranes and promote droplet formation. Hence, the formation of BBMV may be accompanied by changes in the protein components of the inner and outer leaflets of the plasma membrane that may similarly influence surface energy and curvature.

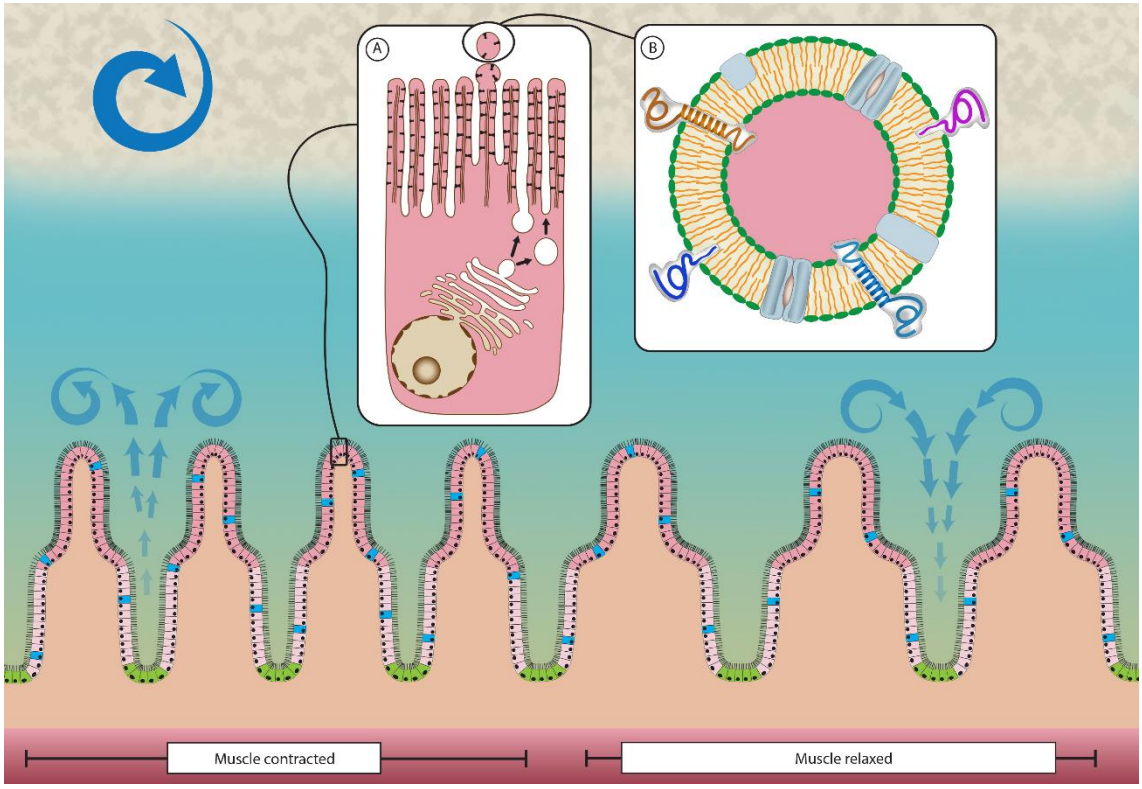

Figure 3. Physiological processes occurring near the brush border

Colour code, from top to bottom: fawn: digesta; aqua: mucus layer; salmon: villi; and fuschia: muscularis mucosae. Colour code, for cells: green: stem cell; pale pink: differentiating cells; pink: mature enterocyte; and blue: goblet cell. Insert B, membrane bilayer: yellow and green; gold and pale blue: brush border enzymes with peptide anchors; dark blue and purple: brush border enzymes with GPI anchors; and grey: membrane proteins. Dark arrows indicate mixing in the centri-luminal compartment; light arrows indicate mixing in the peripheral/peri-mucosal compartment caused by mucosal folding. Inset A shows detail of the mechanisms involved in brush border membrane vesicle production, and detail of the brush border membrane vesicle is shown in inset B.

Structural components of the plasma membrane may also restrict the sites at which either exocytosis or endocytosis can occur. Cytoskeletal elements sterically hinder the invagination of the apical membrane and restrict endocytosis to the bases of microvilli, where budding does not occur (Hansen et al., 2009). Hence, the absorption of dietary lipid, by endocytosis, can only occur at the bases of microvilli via clathrin-coated pits (Hansen et al., 2007). The process of endocytosis temporarily shortens microvilli and changes the proteinaceous components of the villus membrane by selective translocation of integral BB enzymes (Hansen et al., 2007). Immunofluorescence microscopy shows a selective translocation of ALP, but not MGAM, APN, aminopeptidase A (APA, EC

3.4.11.7) or LPH after the endocytosis of lipids (Hansen et al., 2007). Similar restrictions with the site, and change in protein composition, may be involved in the formation of BBMV at the apex.

The linear densities of enterocyte microvilli vary along the length of the villus. Villus density on the apical membranes of immature human enterocytes that are situated close to the germinal layer at the base of villi is lower (4.7 per μm) than on mature enterocytes at the apical tip (10.7 per μm) (Brown, 1962). If the rates at which BBMV are released from microvilli are constant along the long axis of the villus, then the greater density of microvilli at the apex would lead to higher numbers of BBMV being released at this site. This would suggest that the principal site of digestion in the peripheral sub-compartment of the small intestinal lumen (Pappenheimer, 2001, Lentle and Janssen, 2011d) would be around the tips of the villus, provided that there was little convective transfer of fluid between the villi. However, recent findings suggest that there is considerable convective mass transfer from translational movements of the mucosa relative to the muscular layers of the small intestine, causing spaces between villus tips to cyclically decrease (Lentle et al., 2013), see figure 3. Hence, BBMV would likely be dispersed within this sub-compartment regardless of the sites at which they are liberated.

Once they are formed, the movement of BBMVs is driven by diffusion and mixing within the luminal compartment of the intestine. A number of recent studies (Brasseur et al., 2009, Lentle et al., 2013) suggest that mixing takes place in separate functional compartments in the central and peripheral compartments of the small intestine. This does not preclude admixture of secretions from the two compartments; indeed this is required for the normal process of digestion: BB enteropeptidase is necessary for activation of luminal pancreatic proteases (Light and Janska, 1989), and proteases secreted by the pancreas are required for the post-translational processing of some BB enzymes (Hauri et

al., 1979). A substantial proportion of BB enzymes responsible for the digestion of oligomers generated by luminal digestion are (initially at least) bound to BBMV (McConnell et al., 2009). Even though BBMV have been identified in the luminal contents of the small intestine (McConnell et al., 2009) it seems likely that there is a degree of relative retention of BBMV within the peripheral sub-compartment. There are no published data in regards to the relative densities of BBMV at the two sites; however, given that the diffusivity of a particle is inversely proportional to its size (Cu and Saltzman, 2009, Norris et al., 1998) the rate at which BBMV transit from the peripheral to the central part of the lumen would likely be reduced compared with that of soluble BB enzymes, particularly in viscous digesta where convective mixing is reduced.

The adherent and mobile mucin layers on the surface of the mucosa (Cone, 2009) may further restrict advective mixing and increase the minimum length of the diffusion path of BBMV. The permeability of purified mucins to polystyrene microspheres decreases sharply over the size range of 100–300 nm, *i.e.* as they approach the pore size (5-200 nm) of the mucin matrix (Norris and Sinko, 1997). Similar dynamics would be expected to operate with BBMV as the estimated mean diameter of 100 nm (range: 50-200 nm) (Boffelli et al., 1997, Perevucnik et al., 1985, McConnell et al., 2009) is close to this size. However, such restriction would require that the mucosa be covered by a contiguous layer of mucin whereas recent work suggests that mucin masses produced by individual goblet cells do not always fuse together, and may remain as discrete aggregates that allow material to pass between them, in effect acting as a gel diffusion system (Lim et al., 2013). The ability of a particle to diffuse through mucin is also altered by its surface properties. Particles with negative surface charges have greater passage rates compared with those with positive and neutral surface charges (Norris and Sinko, 1997, Crater and Carrier, 2010). As the surfaces of BBMV bear a net negative charge (a surface potential of -21.1

mV) (Ohsawa and Ohshima, 1984) they are likely to repel the negatively charged carbohydrate side-chains of mucins (Strous and Dekker, 1992). This would facilitate the passage of BBMV through a gel mucin matrix, provided the ambient pH and ionic conditions were favorable. Given that a number of hydrophobic micro-particles, such as mixed lipid bile micelles (1-6 μm in diameter), can transit the mucin layer in bulk (Mazer et al., 1979), it does not seem likely that their hydrophobic regions interact significantly with similar regions on the backbone of mucins (Strous and Dekker, 1992). However, bile does alter the electrostatic properties of mixed micelles, and imparts a negative charge that may further facilitate diffusion (Macierzanka et al., 2011).

2.1.4 Soluble BB enzymes

Brush border enzymes may be released from BBMV by a number of methods, some of which may replicate mechanisms *in vivo*. Brush border enzymes with trans-membrane peptide anchors can be cleaved from the membrane by proteolysis resulting in a soluble enzyme (Semenza, 1986). While both peptide and GPI anchored enzymes may be liberated from BBMV by detergents (*e.g.* bile, triton X100, emulphogen BC-720 and dodecyl sulfate). Detergents disrupt the integrity of the BBMV membrane generating amphipathic enzymes (enzymes containing hydrophilic and hydrophobic regions) (Bordier, 1981) because they preserve the membrane anchor during solubilisation. Liberation from the membrane, by either means, may augment or attenuate enzymatic activity (Sigrist et al., 1975, Matsumoto et al., 1995, Auricchio et al., 1963) and alter pH profile, heat stability and kinetics (Matsumoto et al., 1995). For instance, SI, maltase, LPH, and ALP are solubilised when BBMV are exposed to human duodenal secretions (Young and Das, 1990). While SI and maltase remain relatively stable in solution, ALP and lactase progressively lose activity over time (Young and Das, 1990). These effects may result from concomitant changes in the enzyme microenvironment (Ganapathy et al.,

2006, Murer et al., 1976) rather than a greater susceptibility of the solubilised enzyme to further proteolysis.

The manner in which a BB enzyme is anchored to the apical or BBMV membrane appears to determine its susceptibility to cleavage by proteolytic pancreatic enzymes. Enzymes that are anchored to the membrane by a trans-membrane hydrophobic peptide sequence possess a stalked region of variable length (~ 2.0-9 nm) that projects from the apical membrane into the lumen and links to the enzyme proper (Kenny et al., 1983). This region is susceptible to proteolytic cleavage. Enzymes with a short stalked region are less susceptible to pancreatic proteases, a property thought to be due to steric hindrance (Kenny et al., 1983). Treatment of BBMV with the protease papain (smallest dimension 3.7 nm) cleaves all BBMV enzymes with stalked regions greater than 2-3 nm (Kenny et al., 1983). The length of the stalked region varies between BB hydrolases, *e.g.* MGAM 2.5 nm (Norén et al., 1986), NEP 2 nm (Kenny et al., 1983), SI 3.5 nm (Cowell et al., 1986), and APN 5 nm (Hussain et al., 1981). Hence, enteropeptidase can be detached from BBMV by either papain, trypsin or chymotrypsin, while MGAM, LPH, and SI can only be released by papain (Nordström, 1972).

Not all soluble enzymes extracted from mucosal scrapings may be the products of proteolytic cleavage from BBMV. Enzymatically active isoforms of aminopeptidase N (Reisenauer et al., 1992), MGAM and SI (Galand and Forstner, 1974a) that originate in cytoplasmic organelles, or in the cytosol of the enterocyte, may be liberated during BB enzyme purification. For example lysosomal rupture induced by hypotonic conditions (Galand and Forstner, 1974b), and the mechanical disruption of organelles, may liberate soluble enzymes during the normal process of enzyme purification (Maestracci, 1976). Further, enzymes with GPI anchors can be released from the membrane by phosphatidylinositol specific phospholipase C and D (Itami et al., 1997), bacterial

phosphatidylinositol specific phospholipases (Low, 1989), lysophosphatidylcholine (Nakano et al., 2009), bile (Götze et al., 1972) and (to a limited extent) by proteolysis (Louvard et al., 1975, Nordström, 1972). This could be due in part to the structure and glycosylation of the linked section (Low, 1987, Low and Saltiel, 1988). Enzymes with trans-membrane hydrophobic peptide anchors, such as SI and enteropeptidase, can also be liberated from BBMV by bile, though less readily than with proteolytic enzymes (Nordström, 1972). All membrane-bound digestive enzymes appear to be resistant to solubilisation by changes in ionic strength and local pH (Semenza, 1986).

There is suggestion that isoforms of BBMV enzymes also exist in soluble forms in the cytosol of enterocytotes (Seetharam et al., 1977, Galand and Forstner, 1974b). It is suggested that soluble cytosolic (SC) BB enzymes differ from those derived from BBMV by proteolytic cleavage (Galand and Forstner, 1974b). In the suckling rat, SC-neutral MGAM and BBMV-bound MGAM display equivalence in molecular weight, heat sensitivity and kinetics in the digestion of maltose (Galand and Forstner, 1974b). Yet inhibition studies suggest that once BBMV-bound MGAM is solubilised from vesicles by papain proteolysis, it is more sensitive to TRIS inhibition than the reported SC-neutral MGAM and BBMV-bound MGAM (Galand and Forstner, 1974b). This suggests that SC-neutral MGAM may be distinct from soluble MGAM formed by proteolytic cleavage from BBMV (Galand and Forstner, 1974b). However, other reports suggest that SC-BB enzymes are not distinct variants; for example, SC-SI is 40 kDa smaller than the equivalent BBMV-bound enzyme, does not have all its antigenic determinants, and has reduced specific activity (10-50 %) (Cezard et al., 1979). Likewise, a proposed SC-APN is less glycosylated than its BBMV counterpart (Maze and Gray, 1980, Reisenauer et al., 1992) and 40 kDa smaller (Maze and Gray, 1980). Rather than being soluble versions of BB enzymes they may be newly synthesisched versions of BB enzymes liberated from

intracellular membranes during purification. Pulse chase experiments following the sequence of synthesis and processing of APN indicate that the SC form, evident soon after translation, disappeared 30 minutes post-chase (Ahnen et al., 1982). Hence, it is uncertain whether the reported soluble versions of a given BB enzyme result from a separate secretory process or from cellular lysis, the latter liberating nascent BB, lysosomal or microsomal versions. It seems likely that all of these processes can occur.

The relative proportion of soluble, presumably cytosolic, BB enzymes decreases with age (Seetharam et al., 1977, Reisenauer et al., 1992). In the suckling animal, the absorption of nutrient oligomers is augmented by the "leakiness" of the intestinal mucosa, which allows direct uptake of macromolecules via pinocytosis (Williams and Beck, 1969). This leakiness declines, and the specific activity of BBMV-bound hydrolases increases, after weaning (Williams and Beck, 1969). This suggests that soluble BB enzymes have a greater role in the digestion of nutrients at a time when the composition of food is most predictable. Throughout the suckling period the BB of the small intestine undergoes histological and biochemical maturation, causing the proportions of BB architectural proteins and hydrolytic enzymes to change (Seetharam et al., 1977). During post-natal maturation in the rat small intestine, the relative proportion of a reputed 43.5 kDa precursor of actin (Seetharam et al., 1977) increases from 0.2 % of the total BB membrane proteins at 12 days to 31.4 % at 37 days (Seetharam et al., 1977). Further, there are greater proportions of SC-BB digestive enzymes in immature small intestinal mucosa when the proportions of component microvillar cytoskeletal proteins are low (Seetharam et al., 1977). Hence, in the suckling rat, SC-ALP, lactase and MGAM account for 10-25 % of their respective enzyme activity in the proximal, and 45-70 % in the distal small intestine (Seetharam et al., 1977). At weaning, the relative proportion of the enzyme activity by these SC enzymes drops to 2-25 % throughout the small intestine, *i.e.* the source of

activity is primarily from bound enzyme (Seetharam et al., 1977). Similar changes are seen with APN (Reisenauer et al., 1992), *i.e.* the specific activity of SC-APN falls by up to 25 % in the distal small intestine at weaning (Seetharam et al., 1977, Reisenauer et al., 1992). These results suggest that the development of microvillar cytoskeletal proteins, associated with the vesiculation process, appears in synchrony with the decline of the SC enzyme variants and pinocytosis. In the absence of these architectural proteins, BBMV do not vesiculate normally (Tyska et al., 2005, McConnell and Tyska, 2007) and BBMV are not dispersed into the peri–apical space. Hence, direct secretion of SC enzymes may make up for the shortfall.

2.1.5 Cellular expression of BB enzymes

The cellular production of BB enzymes can be regulated at the levels of gene transcription (Krasinski et al., 1994), synthesis (Seetharam et al., 1980), post-translational glycosylation (Beaulieu et al., 1989) and turnover (Seetharam et al., 1980). All levels of regulation are integrated for each protein allowing precise control of enzyme expression and location. Enzyme production rate also varies with the cytological (Seetharam et al., 1977) and anatomical location (Skovbjerg, 1981) of the parent enterocyte both along the axis of the villus, and along the length of the small intestine (Weiser, 1973a). Production, expression and secretion of enzymes at these various sites may be further modulated by a number of ancillary factors, which include diet (Sonoyama et al., 1994, Goda et al., 1995), hormones (Raul et al., 1983, Herbst and Koldovsk, 1972), stage of growth (Henning, 1981) and intensity of local mechanical stimulation (Maestracci, 1976). The modulation of BB enzyme expression is not fully understood, but ligand-mediated transcription factors such as peroxisome proliferator–activated receptors (PPAR)

(Pégorier et al., 2004, Bünger et al., 2007) and caudal related homeobox 2 (Cdx2) /+ genes are implicated.

Brush border enzymes begin to be expressed by enterocytes at some point during their migration from the germinal layer, near the base of the crypt, to the apex of the villus. This migration normally takes 48 to 72 hours in the murine duodenum and jejunum, 24 hours in the murine ileum (Creamer et al., 1961) and around five days in the human small intestine (Marshman et al., 2002, Van Beers et al., 1995a). Further, there appears to be an initial period during enterocyte differentiation when no digestive enzymes are produced, which accounts for the period, of around 14 days, of secondary lactase deficiency after lysis of enterocytes by rotavirus or other pathogens (Heyman, 2006).

The timing of BB enzyme expression during the migration of the enterocyte determines the point along the length of the villus at which enzymatic activity commences. However, the point at which expression commences, and the period over which this expression is maintained, appears to differ between enzymes. The activities of SI and γ-glutamyl transpeptidase (GGT, EC 2.3.2.2) peak around the midpoint of the villus and decline at the tip, in concert with mRNA synthesis (Ferraris et al., 1992, Brown and Sepúlveda, 1985, Cezard et al., 1983, Morrill et al., 1989, Tsuboi et al., 1985, Weiser, 1973b). In contrast, mRNA expression and enzymatic activity of ALP decline from crypt to tip (Ferraris et al., 1992, Brown and Sepúlveda, 1985, Cezard et al., 1983, Morrill et al., 1989, Tsuboi et al., 1985, Weiser, 1973b). This is in keeping with the proposed role of ALP in the absorption of lipids (Hansen et al., 2007) and the detoxification of bacterial lipopolysaccharides (Poelstra et al., 1997, Lalles, 2010), both of which take place in the crypts.

2.1.6 Site specific differences in the specific activities of BB enzymes

The expression of BB enzymes varies among the various segments of the small intestine. The pattern of expression, secretion and activity of the majority of BB enzymes suggest that they are of greater importance in the central and distal small intestine than in the duodenum. One of the few exceptions to this is enteropeptidase, which is only expressed in the duodenum (Louvard et al., 1973). Thus, levels of human SI and LPH protein are highest in the jejunum and are lower in the duodenum and ileum (Skovbjerg, 1981). Similarly, in the rat, the specific activity, mRNA and concentration of LPH protein are highest in the jejunum and decrease proximally and distally (Duluc et al., 1993). On the other hand, trehalase mRNA levels are highest in the duodenum and decrease towards the distal ileum (Oesterreicher et al., 1998), although synchrony between mRNA expression and enzyme activity has not been demonstrated.

The specific activities of the various BB enzymes show similar segmental patterns of variation. Specific activities of rat aminopeptidase P (APP, EC 3.4.11.9) (Matsumoto et al., 1995), APN (Erickson et al., 1992, Yoshioka et al., 1987) and GGT are highest in the jejunum (Ferraris et al., 1992); those of ALP (Ferraris et al., 1992) and ACE decrease from the proximal to the distal small intestine (Erickson et al., 1992, Yoshioka et al., 1987); and those of DPPIV (Erickson et al., 1992, Skovbjerg, 1981), MGAM (Skovbjerg, 1981) and APA increase from the proximal to the distal small intestine (Skovbjerg, 1981). Similarly, in dogs with Thiry-Vella loops (surgically isolated and cannulated sections of intestine used for studying intestinal secretions) the activities of sucrase and maltase are higher in secretions collected from the proximal jejunum (19 and 22.4 mg/cm^2/hr, respectively) than from the distal ileum (2 and 4 mg/cm^2/hr, respectively) (Cajori, 1933). The kinetics of BB digestive enzymes also change with the maturity of the intestinal mucosa. The V_{max} of porcine jejunal BBMV-bound APN, determined *in vitro*, is low

during suckling (7.04 μmol/min/mg), peaks at post-weaning (13.36 μmol/min/mg) and is somewhat lower in the adult (9.5 μmol/min/mg) (Fan et al., 2002). A similar pattern of specific activity is seen with ALP (Fan et al., 2002), while SI specific activity peaks at adulthood (Fan et al., 2002, Reisenauer et al., 1992). These changes may reflect changes in the relative proportions of nutrients in infant and juvenile foods (Fan et al., 2002). However, other workers postulate that changes in the V_{max} of porcine ALP, APN and SI during weaning could result from differences in the extent of post-translational glycosylation (Chu and Walker, 1986, Biol et al., 1991), the expression of different genetic isoforms (notably with ALP) (Engle et al., 1995) or differences in the composition and fluidity of the associated BBMV membrane (Hubner et al., 1988, Schwarz et al., 1984), associated with maturation of the enterocyte.

2.1.7 Effects of mechanical strain

The level of mechanical strain that is applied to the small intestinal mucosa may also influence the genesis and pattern of BB enzyme secretion by mucosal enterocytes. Application of cyclic lateral strain, at physiological magnitudes and frequencies (10 cpm) to monolayer cultures of human CaCo-2 cells promoted cellular proliferation and differentiation (Basson et al., 1996), while stimulating synthesis of dipeptidylpeptidase and inhibiting synthesis of ALP (Basson et al., 1996). The frequency of cyclical strain necessary to induce cellular proliferation varied with tissue type; enterocytes responded best to a frequency of 10 cpm (Basson et al., 1996), vascular endothelial cells to a frequency of 60-90 cpm (Frangos et al., 2001) and osteoblasts to 1-6 cpm (Matsuda et al., 1998). These differences presumably reflect the frequencies in biological systems that govern strain, such as the slow wave induced contractions of longitudinal muscle in the small intestine (Lentle and Janssen, 2011e). In the small intestine, differences in the

apparent viscosity and pseudoplasticity of digesta will dictate the degree of stretch applied to segments of the small intestine (Lentle and Janssen, 2011c).

Morphological characteristics of mucosal components also change with sustained longitudinal strain. There is a significant increase in mucosal thickness, crypt depth and surface area when longitudinal stretch is maintained *in vivo*, for a period of seven days, on a loop of porcine jejunum, (Spencer et al., 2006). Hence, some of the decrease in villus height and crypt depth that is reported during starvation (Hernandez et al., 1999) may result from a general reduction in longitudinal strain caused by a lack of luminal distension.

2.1.8 Effects of diet

The relative proportions of various types of BB enzyme activity in the various segments of the small intestine are influenced by the relative proportions of macronutrients in the diet (Tanaka et al., 2008, Goda et al., 1995, Ferraris et al., 1992). In mice, the specific activity of SI is consistently higher in the duodenum and proximal jejunum following administration of a high carbohydrate/low protein diet than it is in mice that have received a no carbohydrate/high protein diet (Ferraris et al., 1992). Conversely, the specific activities of GGT and ALP from the duodenum and jejunum, are greater in mice that had received a no carbohydrate/high protein diet than in those fed a high carbohydrate/low protein diet (Ferraris et al., 1992). However, the expression of BB enzymes does not always appear to be substrate specific. The expression of LPH mRNA is up-regulated after increases in non-substrate oligosaccharides, such as sucrose, and with fructose and glycerol, the products of general digestion (Tanaka et al., 1998).

The mechanisms that underlie the changes in gene expression in response to changes in dietary composition are not fully elucidated. However, increases in dietary carbohydrate content and decreases in fat content are known to induce alterations in the pattern of

histone acetylation in the transcription and promoter regions of the SI gene, resulting in its up-regulation (Honma et al., 2007). Further evidence suggests that signaling by gastrointestinal taste receptors may drive such gene modulation (Margolskee et al., 2007, Shirazi-Beechey et al., 2011).

2.1.9 Effects of starvation and hypothyroidism

Phenotypic changes during periods of starvation similarly indicate that gene expression may change with nutrient intake. However, the directions of these changes do not always reflect the availability of particular macronutrients (Hodin et al., 1995). Intracellular concentrations of ALP (Hodin et al., 1995), SI and APN mRNA (Galluser et al., 1991) are all reduced during starvation whilst the rate of LPH mRNA synthesis is increased (Hodin et al., 1995, Galluser et al., 1991). Conversely, in hyperthyroidism ALP mRNA expression is increased whilst lactase mRNA expression is decreased (Hodin et al., 1995, Watson and Tuckerman, 1971). It is important to note that the relationship between gene expression and the concentrations of active enzymes within the lumen during starvation may be confounded by villus atrophy, and consequent changes in the overall number and the relative proportion of mature and immature enterocytes. Increased expression and secretion may not necessarily increase overall output per villus.

2.2 Enzymatic activity

2.2.1 Nomenclature

The nomenclature of BB enzymes is not always substrate specific as enzymes often have multiple activities (Gray, 2000). For example, sucrase (EC 3.2.1.48) is named on the basis of its ability to cleave the α1,β2 bond between the component monosaccharides, but it is also able to cleave the α1,4 linkages of the smallest glucosyl oligosaccharides, *i.e.* maltose and maltotriose (Van Beers et al., 1995a). This confusion has in part arisen from the fact

that allied groups of BB enzymes, and separate sites on a given enzyme, appear to work synergistically on nutrient oligomers. Indeed, such synergy may also account for the continued association of the component enzymes with the BB membrane, allowing them to simultaneously process the substrate.

2.2.2 Substrate specificity: relationship between BB and pancreatic enzymes

A range of gastric and pancreatic enzymes act on chyme in the gastric and small intestinal compartments to hydrolyze various dietary nutrients. Broadly speaking, this action causes the polymeric structures of protein and carbohydrate to be converted to oligomers and the physical mass of fat droplets to be reduced to micellar form in the intestinal lumen (Beck, 1973, Maldonado-Valderrama et al., 2011). The specificities of enzymatic cleavage by pancreatic and gastric enzymes necessarily differ from those of BB enzymes. A lesser range of linkage specificity is required to convert polymers to oligomers than is required to cleave the entire range of linkages that are found in oligomers to component monomers. Hence, the actions of BB enzymes can be considered to complement gastric and pancreatic enzymatic actions by reducing the oligomers produced from macronutrients to monomers. The best example of this complementary activity is achieved by a sub-set of BB exopeptidases that hydrolyze *N*-terminal amino acids, a specificity that is absent from the pancreatic peptidases (Beck, 1973).

It is useful to consider the BB enzymes as a series of consortia based on the macronutrients that they digest, and on their differences from their pancreatic and gastric counterparts. However, it is important to remember that there may be interaction between substrates that require the synergistic action of these consortia. For example, the hydrolysis of lipids from mixed micelles is improved when proteolytic enzymes

hydrolyze and liberate hydrophobic oligopeptides that partition into the oil-water interface (Lueamsaisuk et al., 2013).

The digestion of carbohydrates by pancreatic secretions is limited to the action of a single *α*-amylase. The active site of this endoenzyme locates to sequences of five consecutive α1,4 glucose units in starches and cleaves them between the second and third units (Quesada-Calvillo et al., 2006). Thus, it has nominal ability to hydrolyze the α1,4 linked glucose moieties of maltose and maltotriose and is unable to hydrolyze at or near the α1,6 linkages of amylopectin (The UniProt Consortium, 2014, Bird and Hopkins, 1954, Beck, 1973). The resulting assortment of oligosaccharides (maltose, maltotriose and *α*-dextrins) are hydrolysed by a consortium of BB enzymes (Beck, 1973). The oligosaccharidases (SI, MGAM and LPH) work in concert to cleave the glycosidic bonds of these oligomers, namely α1,4 linkages (maltase, glucoamylase, sucrase and isomaltase), α1,6 linkages (isomaltase and to a lesser extent glucoamylase), α1,β2 linkages (sucrase), and β1,4 linkages (lactase and phlorizin hydrolase). The α1,α1 linkages of the disaccharide trehalose are found in a small selection of foods: namely mushrooms, yeast, insects (Van Beers et al., 1995a), honey, lobster, prawns, mirin and sherry (Richards et al., 2002). Trehalose is hydrolyzed by the BB enzyme trehalase (Richards et al., 2002).

The actions of pancreatic peptidases differ from those of BB enzymes. Namely, pancreatic peptidases include serine endopeptidases, with specificity for internal amino acid residues and carboxypeptidases with specificity for *C*-terminal residues (Beck, 1973). The pancreatic endopeptidases all belong to the serine S1 protease family that have a serine residue at the active site, but the substrate specificity of each enzyme differs (Hedstrom, 2002). For example, trypsin has specificity for arginine and lysine at the P_1 site (the hydrolyzed bond being termed P_1-P'_1) (Perona and Craik, 1995, Harris et al., 2000). Chymotrypsin has specificity for hydrophobic residues at the P_1 site, *i.e.* tyrosine,

phenylalanine, tryptophan, methionine and leucine (Harris et al., 2000, Stevens, 2006). Elastase prefers neutral aliphatic amino acids at the P_1 site, *e.g.* serine, leucine, alanine and valine residues (Naughton and Sanger, 1961, Stevens, 2006). The action of all of these enzymes is inhibited by a proline at the P'_1 site on the substrate (Stevens, 2006).

Pancreatic carboxypeptidases are able to reduce some of the oligopeptides that are generated by the action of the serine endopeptidases by exopeptiditic cleavage of residues at the *C*-terminal. Carboxypeptidase B preferentially cleaves lysine and arginine residues (Beck, 1973, Stevens, 2006), but not histidine (Folk and Gladner, 1958) or proline (Stevens, 2006). Carboxypeptidase A prefers aromatic/aliphatic residues, *e.g.* leucine, isoleucine, alanine and valine, but hydrolyzes most amino acids except those with a *D*–configuration, proline, sarcosine (Stahmann et al., 1946) or the charged groups such as arginine, lysine, glutamic acid and aspartic acid (Beck, 1973, Stevens, 2006).

The action of pancreatic proteases is therefore likely to produce a high proportion of small peptides (Beck, 1973) and oligopeptide residues with proline rich interiors and proline on their carboxy terminals. A consortium of BB oligopeptidases, with diverse specificities, is required to digest these oligopeptide products. The largest group, BB *N*-terminal exopeptidases, have specificity for *N*-terminal dipeptides and/or amino acids; including DPPIV, and GGT, and the aminopeptidases, APN, APA and APP (Amidon and Lee, 1994). A smaller group of BB exopeptidases specifically cleaves *C*-terminal dipeptides (*e.g.* ACE) and *C*–terminal amino acids (*e.g.* carboxypeptidase P (CPP, EC 3.4.17.16)) (IUBMB, 2013). A group of BB endopeptidases hydrolyzes small oligopeptides; this group includes NEP (Amidon and Lee, 1994), meprin A subunit β (MEP, EC 3.4.24.63) and enteropeptidase. The action of these groups is complemented by the BB dipeptidases that hydrolyze dipeptides to component amino acids (*i.e.* DP1). Those oligopeptides that are not hydrolyzed by BB peptidases may be absorbed as di- and tri-peptides *via* the

relevant proton co–transporter for subsequent hydrolysis in the cytosol (Daniel, 2004, Leibach and Ganapathy, 1996).

There is less diversity within the lipolytic BB consortium. This is presumably as a consequence of the efficient digestion of lipids to component fatty acids, by a comprehensive suite of pancreatic enzymes within the lumen (Lowe, 2002). Pancreatic secretions are the principal small intestinal source of enzymes that digest triacylglycerol, in conjunction with bile and colipase. These enzymes are PTL and BSAL (Brownlee et al., 2010, Beck, 1973). Pancreatic triacylglycerol lipase hydrolyzes lipids displaying preference for the sn1 and sn3 positions (IUBMB, 2013, Brownlee et al., 2010). On the other hand BSAL shows no positional specificity towards lipids, hydrolyzing mono, di and triacylglycerol as well as cholesterol esters, phospholipids, lysophospholipids and ceramides (Hui and Howles, 2002, Wang and Hartsuck, 1993, Bauer et al., 2005). Similarly, the bulk of dietary phospholipids are digested by pancreatic PLA2 (Beck, 1973). The latter activity appears to be augmented by PLB1, the principal BB phospholipase, and PLA2 group IVC (EC 3.1.1.4) (McConnell et al., 2011). However, some products of luminal digestion may require further hydrolysis by BB enzymes before being absorbed (Hansen et al., 2007, Iqbal and Hussain, 2009). One class of lipids that is not degraded by pancreatic enzymes are sphingolipids. Brush border alkaline sphingomyelinase (AS, EC 3.1.4.12) and neutral ceramidase (NS, EC 3.5.1.23) are responsible for the hydrolysis of this dietary class (Vesper et al., 1999). Both phospholipids and sphingolipids are found in small amounts in most food, but are particularly abundant in eggs, soybeans, cream and cheese (Vesper et al., 1999, Wehrmüller, 2008)

The role of ALP in the small intestine is subject to ongoing debate. Its roles in digestion are thought to be in the digestion of phosphate monoesters (Cerneus et al., 1993) as well

as regulating the absorption of lipids (Hansen et al., 2007, Narisawa et al., 2003). Other proposed roles include acting as a phosphate binding protein (Roubaty and Portmann, 1988) and maintaining the pH microclimate by regulating local alkalinity in the face of luminal acidity (Mizumori et al., 2009, Akiba et al., 2007).

2.2.3 Problems in quantifying the activities and kinetics of BB enzymes

The principal means by which the quantities of BB enzymes that are produced in the various sites and segments of the intestinal mucosa have been estimated is by comparisons of their specific activities. For such comparisons to be valid, due regard must be paid to the method of harvest (Kessler et al., 1978, Schmitz et al., 1973, Kim et al., 1972), the timing of harvesting, (Stevenson et al., 1975, Ferraris et al., 1992, Saito et al., 1976), diet prior to harvesting (Raul et al., 1987, Goda et al., 1995), age (Fan et al., 2002), nutritional status (Mahmood et al., 1984) and the frequency of feeding (Farooq et al., 2004) of the animal from which the sample is obtained. The method by which enzymatic activity is characterized is further complicated by the technique used for standardisation. It is possible to standardise enzymatic activity by relating it to BBMV protein (Fan et al., 2002), purified BB enzyme protein, purified soluble enzyme protein (Maze and Gray, 1980, Kim et al., 1972), SC enzyme protein (Timofeeva et al., 2002) or to the overall protein concentration in the mucosal homogenate (Tarvid, 1991). However, these values may not be directly comparable as they will include different fractions of BB and cytosolic enzymes as well as different proportions of total protein.

The choice of assay conditions can complicate comparisons between studies. The ambient pH, substrate, buffering, ionic conditions and temperature (Auricchio et al., 1965, Kolínská and Kraml, 1972, Vasseur et al., 1982) will depend on the site at which the enzyme is presumed to function. These conditions vary considerably over the range of

sites in which BB enzymes are found. The ambient pH is around 7.4 near the pancreatobiliary opening (Hong et al., 1967), and approximately 6.1 and 7.3 in the peri–apical space of the proximal jejunum and distal ileum, respectively (Lucas, 1983). Additionally, there is a pH gradient from crypt to tip along the length of the villus within each segment, the pH being lower at the apices of the villi, where enterocytes are capable of secreting H^+ ions, than in the crypts at their bases (Daniel et al., 1989). The pH is reported to vary from villus tip to crypt between 6.85 and 7.11 in the duodenum, between 6.65 and 7.06 in the jejunum and between 7.26 and 7.33 in the ileum respectively (Daniel et al., 1989). The pH of the microenvironment surrounding BBMV may be modified by the operation of a number of apical transporters that continue to function within the BBMV membrane (Kaunitz and Wright, 1984) generating ionic species that influence local pH (Wistrand and Kinne, 1977), *e.g.* the sodium/proton antiporter (Murer et al., 1976, Ganapathy et al., 2006).

The electrolyte environment of BB enzymes may also influence their activities. The SI hydrolysis of sucrose is augmented by 30-40 % in the presence of sodium ions (50 mM) (Sigrist et al., 1975, Auricchio et al., 1963), which decreases the optimal pH of SI from 6.7 to 5.9 (Kolínská and Kraml, 1972). This level of sodium activation *in vitro* may reflect normal ambient conditions in the intestine, *i.e.* in the jejunum the sodium concentration is approximately 142 mM (Lindahl et al., 1997, de Beer et al., 1935). Other BB enzymes are less sensitive in this respect. Variation in ambient levels of either potassium or sodium have no influence the activity of MGAM (Sorensen et al., 1982).

Kinetic parameters of BB enzymes have been determined in many studies. The substrate affinities (K_m) of BB enzymes are described by the Michaelis constant: the substrate concentration that gives a reaction rate half of the enzyme maximum rate V_{max} (Cornish - Bowden, 1976). For pancreatic enzymes the K_m ranges between 15.6 mM (Lowe, 1992)

to 1.2 x 10^{-2} mM for those of low and high affinity respectively (Anderson et al., 1981). This is similar to the range for the hydrolysis of common substrates by BB enzymes, *e.g.* 19 mM (Gray et al., 1979) to 3.9 x 10^{-3} mM (Halsted et al., 1998). The enzyme turnover rate, $k_{cat,}$ measures the quantity of substrate that is converted to product per second and is the ratio of the Michaelis constant to the enzyme concentration, *i.e.* $k_{cat} = V_{max}/[E]$). This too ranges widely, from 11.6 s^{-1} (Mikhailova and Rumsh, 2000) to 709 s^{-1} (Erickson et al., 1992) for BB enzymes; and from 4.89 s^{-1} (Largman, 1983) to 1.0 x10^4 s^{-1} (Spilburg et al., 1977) for pancreatic enzymes. The final commonly determined kinetic parameter is catalytic efficiency ($k_{cat}/K_{m)}$; catalytic efficiency is determined by the rate in $M^{-1}.s^{-1}$ at which the enzyme acts on it once the substrate has diffused to and bound to it. Brush border enzymes vary between 4.86 x10^2 (Mikhailova and Rumsh, 2000) and to 9.9 x10^5 $M^{-1}s^{-1}$ (Erickson et al., 1992); pancreatic enzymes have a similar range between from 2.12 $M^{-1}.s^{-1}$ (Hui et al., 1993) to 4.83 x 10^6 $M^{-1}s^{-1}$ (Anderson et al., 1981). In summary, comparisons of the various kinetic parameters of pancreatic and BB enzymes (table 49 and table 50 of appendix 1) do not indicate any broad distinctions between the two. However, comparisons on a basis of functional groups are more revealing, as discussed below.

2.3 Kinetic activities of functional groups of BB enzymes

Brush border digestive enzymes are all hydrolases. Analysis of BB hydrolases in a murine BBMV proteome identified 43 % of the spectral counts as BBMV oligosaccharidases, 47 % as peptidases and 10 % as lipolytic enzymes (McConnell et al., 2011). These spectral counts are not a direct reflection of enzyme proportions; larger proteins will yield more peptide fragments, and so elicit more spectra, and some peptide fragments are more easily recorded and identified than others. No doubt the ratios of the enzymes that constitute

each consortium will vary with location in the intestine, diet, age and other factors. However, spectral counts can provide some impression of the distribution of the enzymes acting on the major substrates. The component enzymes that comprise these consortia are discussed below.

2.3.1 Oligosaccharidase consortium

All BB border oligosaccharidases are exoenzymes (Van Beers et al., 1995a) that are able to catalyze the cleavage of more than one type of linkage. While there is some overlap in their various activities, a number have a unique ability to cleave a particular linkage. There are three α-glucosidases and one β-glycosidase (Semenza, 1986). The α-glucosidases include SI, MGAM and trehalase; LPH is the sole β-glycosidase.

Maltase-glucoamylase

The maltase–glucoamylase complex accounts for about a third of the spectral counts identified as BB oligosaccharidase protein in the murine BBMV proteome (McConnell et al., 2011). Maltase-glucoamylase is a single pass type II integral membrane protein of the glycosyl hydrolase 31 family (The UniProt Consortium, 2014). Maltase–glucoamylase is an α-glucosidase complex that can remove terminal glucose units and does not replicate the endoenzymatic substrate engagement requirements of pancreatic α-amylase (Quesada-Calvillo et al., 2006). It is thought to contribute about 20 % of maltase, a few per cent of isomaltase and all of terminal glucoamylase activity observed (Galand, 1989, Van Beers et al., 1995a). Maltase-glucoamylase has two active sites, the maltase (EC 3.2.1.20) site catalyzes the hydrolysis of terminal, non-reducing α-1,4-linked-D-glucose residues to release α-D-glucose (IUBMB, 2013), and the glucoamylase site (EC 3.2.1.3) catalyses the hydrolysis of terminal α-1,4 linked α-D-glucosidic bonds successively from the non-reducing ends of starch side chains to release β-D-glucose. The

distinctive feature of the latter site is that it can also cleave α-1,6 glycosidic bonds when the next residue is α-1,4 linked (IUBMB, 2013, Van Beers et al., 1995a).

The maltase and glucoamylase sites are able to digest similar substrates, and the two sites have more or less equal affinity. Isolated human recombinant maltase has a K_m for the hydrolysis of maltose of 6.17mM (pH 4.8) a value similar to that of isolated recombinant human glucoamylase albeit at a higher pH (K_m 5.53mM at pH 7) (Ren et al., 2011). However, the maltase site appears to be able to hydrolyze maltose more rapidly than the glucoamylase site. The k_{cat} of the maltase site (47.76 s^{-1} at pH 4.8) is more than twice that of the glucoamylase site (k_{cat} 21.99 s^{-1} at pH 7) (Ren et al., 2011). The k_{cat} /K_m ratios of the maltase and glucoamylase sites are 7.74 x 10^3 and 3.98 x 10^3 $M^{-1}.s^{-1}$ (pH 4.8 and 7 respectively) (Ren et al., 2011) suggesting that maltase may be catalytically more efficient at hydrolyzing maltose. However, it is likely that enzyme activity varies with pH.

The differences in the kinetics of the component enzymatic sites of MGAM are matched by differences in the stabilities of their tertiary structures, which are exemplified by their differing sensitivity to heat. Studies using sodium dodecyl sulfate polyacrylamide gel electrophoresis, show that rat MGAM undergoes slow dissociation with increasing heat, but that enzymatic activity is retained in the intermediate species, *i.e.* there is ongoing enzyme activity in the dissociation products (Flanagan and Forstner, 1979). Maltase activity is inactivated at 76°C and glucoamylase activity is inactivated at 64°C (Auricchio et al., 1965, Kolínská and Kraml, 1972, Kelly and Alpers, 1973). As MGAM is 30-40 % glycosylated (Naim et al., 1988b, Kelly and Alpers, 1973) it is considered likely that these carbohydrate moieties afford MGAM greater structural integrity and heat stability (Kingsley et al., 1986, Vaňková et al., 1994).

The efficiencies of the component sites of MGAM may increase on enzymatic cleavage. The K_m for the digestion of maltose at pH 6 by human MGAM is 3.84 mM, after treatment

with papain (Kelly and Alpers, 1973). This value is lower than those reported for the isolated subunits of recombinant human MGAM, notably maltase 6.17 mM at pH 4.8 and glucoamylase 5.37 mM at pH 7 (Ren et al., 2011). It is noteworthy that the optimal pH of complete human MGAM (pH 6) (Auricchio et al., 1965, Kelly and Alpers, 1973) does not change after solubilisation with papain (Auricchio et al., 1965). The reduced affinity of the isolated MGAM subunits compared to the intact protein may result from the dissociation of the subunits, or a structural change with the loss of membrane association; but again pH differences may have an effect. Certainly, MGAM appears to be less resistant to inhibition following enzymatic solubilisation by papain; when the maltose hydrolyzing capability of BBMV-bound MGAM and papain solubilised MGAM are compared we see that the latter displays greater sensitivity to inhibition by TRIS, the K_i values being 9.5 mM and 2.5 mM respectively (Galand and Forstner, 1974b).

Sucrase–isomaltase

Sucrase–isomaltase is a single pass type II integral membrane protein of the glycosyl hydrolase 31 family (The UniProt Consortium, 2014). The SI complex accounts for around 40 % of spectral counts identified as BB oligosaccharidase protein in the murine BBMV proteome (McConnell et al., 2011). The enzyme can cleave both α-1,4 and α-1,6 linked components in oligosaccharides (Sim et al., 2010), and is composed of two sub-units each with a distinctive active site (Semenza, 1986), sucrase and isomaltase (EC 3.2.1.10). The isomaltase site hydrolyzes terminal α-1,4 glucose from small oligomers, and α-1,6-linked glucose from starch, glycogen and small oligosaccharides with the α-1,6 linkage (IUBMB, 2013). Hence, isomaltase activity complements that of glucoamylase. The sucrase site can hydrolyze small α-1,4 linked oligomers and is the only enzyme

capable of hydrolyzing sucrose (α1,β2 linkages) (Van Beers et al., 1995a, Semenza, 1986).

The complementary activity of SI and MGA originates from a common ancestry. The two enzymes evolved from a common ancestral gene that underwent tandem duplication followed by further duplication that resulted in two structurally comparable enzymes, each with two distinct active sites (Nichols et al., 2003, Sim et al., 2008). While the sub-units within each enzyme are 40 % structurally identical (Sim et al., 2008) there is greater homology between the sub-units of each enzyme. The *N*-terminal sub-units of maltase (*N*-terminal MGAM) and isomaltase (*N*-terminal SI), and the *C*-terminal subunits glucoamylase (*C*-terminal MGAM) and sucrase (*C*-terminal SI), are 60 % structurally homologous respectively (Sim et al., 2008). The activity of these enzymes diverged slightly over time resulting in MGAM and SI displaying mutual specificity towards α1,4 oligosaccharides while SI specificity diverged to include branched α-1,6 oligosaccharides (Sim et al., 2010). This is in response to mutations in the active site of SI that led to differences in the substrate binding pattern and specificity, *i.e.* a mutation in the active site of isomaltase led to a hydrophobic +1 sub-site that affects the binding of substrates (Sim et al., 2008).

The kinetics of SI are contradictory. This may be due to differing enzymatic forms, *e.g.* BBMV-bound, proteolytic, detergent solubilised or autolysed enzyme (the spontaneous separation of SI from BBMV), modifying activity. Autolyzed and subsequently purified rat SI had a K_m of 19 mM and k_{cat} of 120 s^{-1} for the hydrolysis of sucrose and a K_m of 5 mM and a k_{cat} of 74 s^{-1} for the hydrolysis of isomaltose (pH 6.1) (Gray et al., 1979). This suggests that SI has a lower substrate affinity but a greater conversion rate with a sucrose substrate than isomaltose (Gray et al., 1979). However, the efficiency of isomaltose

digestion by SI (k_{cat}/K_m 1.48 x 10^4 $M^{-1}.s^{-1}$) appears to be greater than that of sucrose (k_{cat} /K_m 6.3 x10^3 $M^{-1}.s^{-1}$) at this pH (Gray et al., 1979).

Substrate affinity does not appear to vary between the BBMV-bound or solubilised forms of SI and the isolated sucrase subunit. Hence, BBMV-bound porcine SI has a K_m of 16.57 mM with a sucrose substrate at pH 6 (Fan et al., 2002); autolyzed and purified porcine SI has a K_m of 19.2 mM, with sucrose at pH 6.8, and the purified sucrase subunit from the same source has a K_m of 14.6 mM under the same conditions (Rodriguez et al., 1984). These findings suggest that the affinity of the sucrase site are unaffected by incorporation into the surface membrane of a BBMV, or by other components of the enzyme, bearing in mind that the pH differed between the two studies. Further, these results suggest that the anchoring of SI to BBMV does not sterically hinder the approach of its substrate by diffusion.

These conclusions are not supported by a study that investigated changes in k_{cat} and k_{cat}/K_m following the spontaneous autolysis of SI from the membrane and dissociation into its component subunits, sucrase and isomaltase. Although autolyzed and purified rat SI had the same substrate affinity for sucrose (K_m 19 mM at pH 6.1) as the sucrase subunit alone (19 mM at pH 6.1), it exhibited faster substrate turnover (k_{cat} 120 s^{-1} at pH 6.1) and greater catalytic efficiency (k_{cat} /K_m 6.3 x10^3 $M^{-1}.s^{-1}$ at pH 6.1) than did the subunit (k_{cat} 15 s^{-1} and k_{cat} /K_m of 7.9 x10^2 $M^{-1}.s^{-1}$ at pH 6.1) (Gray et al., 1979). The 8-10 fold reduction in sucrose turnover (k_{cat}) and catalytic efficiency (k_{cat} /K_m) when the sucrase subunit was separated from the isomaltase subunit suggests that catalytic efficiency is decreased when the dimeric structure is lost. It is also noteworthy that the K_m for detergent solubilised and purified rabbit SI (8.4 mM) is very similar, at pH 6.8, to that which has been proteolytically solubilised (8.5 mM) (Sigrist et al., 1975). Judging by both of these examples it appears that solubilisation, either by autolysis or other cleavage, had little

effect on substrate affinity but the speed and efficiency of the enzyme may be altered by changes in structure or the loss of a subunit.

Lactase-phlorizin hydrolase and trehalase

Lactase-phlorizin hydrolase is a single pass type I integral membrane protein from the glycosyl hydrolase 1 family (The UniProt Consortium, 2014). Spectral counts identified as LPH account for a sixth of BB oligosaccharidase protein identified in the murine BBMV proteome (McConnell et al., 2011). Lactase-phlorizin hydrolase is a β-glycosidase complex with two sub-sites. The lactase (EC 3.2.1.108) site hydrolyzes lactose, cellobiose/triose, and some activity towards cellulose and aryl β-glycosides; the phlorizin site (EC 3.2.1.62) hydrolyzes aryl β-glycosides and glyceroceramides (Semenza, 1986) and displays some lactase activity (IUBMB, 2013). The K_m of Triton-X100 solubilised and purified rat LPH is 16 mM, k_{cat} 47 s^{-1} and the k_{cat} /K_m 2.9 x10^3 $M^{-1}.s^{-1}$ (lactose, pH 6) (Mackey et al., 2002). No other species comparable kinetic data regarding the purified subunits or membrane bound form were found, but other available data are displayed in Table 49 of appendix 1.

Trehalase is from the glycosyl hydrolase 37 family (The UniProt Consortium, 2014) and accounts for 4 % of spectra identified as BB oligosaccharidase protein in the murine BBMV proteome (McConnell et al., 2011). The lack of data on trehalase may reflect the minor role it plays in human digestion as it hydrolyzes only trehalose (Van Beers et al., 1995a), a carbohydrate which is low in the western diet (Richards et al., 2002). Triton-X100 solubilised and purified rat trehalase is reported to have a K_m of 10 mM (Riby and Galand, 1985) and 5.4 mM (Sasajima et al., 1975) with a trehalose substrate at pH 6.8 and 5.7, respectively. The optimal pH is reported to lie between 5.5 and 5.7 (Sasajima et

al., 1975). Hence, an increase in pH appears to results in a reduction in the affinity of trehalase for its substrate.

2.3.2 Oligopeptidase consortium

The BB peptidases, which include both exo and endopeptidases, vary widely in their specificity for amino acid residues. The exopeptidases show specificity for either the *C* or the *N*- terminal of oligopeptides and generally produce single amino acids or dipeptides (Amidon and Lee, 1994). In contrast, endopeptidases hydrolyze non-terminal amino acids in oligopeptides of 30 or fewer amino acid residues (Amidon and Lee, 1994, The UniProt Consortium, 2014). The dipeptidase group hydrolyzes dipeptides to their component amino acids.

N-terminal exopeptidases

The *N*-terminal exopeptidases are the most abundant group of oligopeptidases present in the BB (Amidon and Lee, 1994). Enzymes from this group include DPPIV, which hydrolyzes *N*–terminal dipeptides with preference for proline at the P_1 site, in situations where there is no proline or hydroxyproline at the P'_1 site (The UniProt Consortium, 2014). Dipeptidylpeptidase IV has a pH optimum between 7.4 and 8 (Lojda, 1979).

Aminopeptidase P hydrolyzes the release of any *N*–terminal amino acid from di, tri and oligopeptides that are linked to proline (IUBMB, 2013). It has a pH optimum between 8 and 8.2 (Matsumoto et al., 1995).

Aminopeptidase A catalyzes the hydrolysis of acidic amino acid residues such as those of glutamate and aspartate (The UniProt Consortium, 2014). Its pH optimum is between 6.5 and 7 (Danielsen et al., 1980a).

Of the *N*-terminal exopeptidases, APN is the most abundant, accounting for 40 % of spectral counts identified as BB peptidase protein in the murine BBMV proteome with

APA, DPPIV and APP accounting for 13, 6, and 1 %, respectively (McConnell et al., 2011). APN is a member of the M1 peptidase family (The UniProt Consortium, 2014), which require a zinc ion for catalytic activity (Rawlings et al., 2012). The principal action of APN is the cleavage of neutral aliphatic amino acid residues (Benajiba and Maroux, 1980), but the enzyme is capable of hydrolyzing a wide range of substrates, including those containing proline (The UniProt Consortium, 2014), and those with charged residues, (Benajiba and Maroux, 1980), but with reduced activity. Aminopeptidase N releases the *N*-terminal amino acid from an amide or arylamide (The UniProt Consortium, 2014) where the P_1 residue is preferentially a neutral amino acid such as alanine or leucine (Bai and Amidon, 1992). However, a dipeptide is released when the penultimate amino acid (P_1) is proline (The UniProt Consortium, 2014). The optimal pH of human APN is 7.5 with leucine–β–napthylamide as a substrate (Caporale and Troncone, 1988), which is similar to that of porcine and rabbit APN (~pH 7.5) assayed with a alanine *p*–nitroanilide substrate (Feracci et al., 1981).

The kinetic activity of APN varies between species. Triton-X100 solubilised and purified human APN has a K_m of 52 mM and a V_{max} of 59.5 μmol/min/mg with a substrate of leucine–*β*–napthylamide at pH 7.5 (Caporale and Troncone, 1988) while Triton-X100 solubilised and purified rat APN has a much higher affinity, K_m of 0.08 mM, with the same substrate and pH (Reisenauer et al., 1992). Such interspecies variation in kinetics may reflect variation in ambient luminal conditions at the site of action, perhaps according to the relative proportions of nutrients and micronutrients in the diet.

C-terminal exopeptidases (carboxypeptidases)

Pancreatic carboxypeptidases are unable to hydrolyze a number of important amino acid residues. Carboxypeptidase A is unable to hydrolyze those amino acids with charged

groups, *i.e.* arginine, lysine, glutamic acid, aspartic acid (Beck, 1973, Stevens, 2006), *C*-terminal proline, sarcosine or amino acids in the *D*-conformation (Stahmann et al., 1946). While CPB is unable to hydrolyze histidine amino acid residues (Folk and Gladner, 1958), and neither CPA or CPB can efficiently hydrolyze amino acids with proline at P_1 (Stevens, 2006). In the face of this shortfall two groups of BB carboxpeptidase enzymes exhibit complementary specificity. The first comprising those enzymes with a capability of hydrolyzing prolyl residues, the second comprising those that are able to hydrolyze amino acids with acidic R-groups. The proline hydrolysing carboxypeptidases include ACE and CPP. Angiotensin converting enzyme is a single pass type I membrane *C*-terminal exopeptidase, of the peptidase M2 family, that converts angiotensin I to angiotensin II (The UniProt Consortium, 2014), and is also involved in the luminal digestion of dietary protein (Yoshioka et al., 1987). Angiotensin converting enzyme hydrolyzes dipeptides from the *C*-terminal of oligopeptides where P'_1 is not proline, and P'_2 is neither aspartic acid nor glutamic acid (The UniProt Consortium, 2014), but can rapidly release such dipeptides when the P'_2 amino acid is proline (Yoshioka et al., 1988). Angiotensin converting enzyme has an optimal pH of 8.3-8.5 with Bz-Gly-His-Leu as a substrate (Yoshioka et al., 1987). Brush border CPP shows complementary activity as it is able to hydrolyze *C*–terminal amino acids with P_1 proline (IUBMB, 2013, Yoshioka et al., 1988). The pH optimum of CPP is 8.2 with a Gly–Pro–Hyp substrate (Matsumoto et al., 1995). Hence ACE, and CPP act in concert to hydrolyze *C*-terminal prolyl peptide bonds in either of the two positions (Yoshioka et al., 1988).

Kinetic studies confirm that ACE has a preference for substrates with a *C*-terminal proline at the P'_2 position. Triton X-100 solubilised/purified BB ACE has a 1.2–12 fold higher affinity (lower K_m) and 6–9 fold higher catalytic efficiency (k_{cat}/K_m) for substrates with *C*-terminal proline residues than those with *C*-terminal leucine or glycine residues

(Erickson et al., 1992). The catalytic activity of ACE also appears to change with solubilisation. The hydrolysis of Bz-Gly-His-Leu by rat Triton X-100 solubilised/purified ACE at pH 8.5 has a K_m of 1.9 mM, V_{max} of 108 μmol/min/mg, k_{cat} of 719 s^{-1} and k_{cat}/K_m of 1.6 x 10^5 $M^{-1}.s^{-1}$ (Erickson et al., 1992). In comparison BBMV-bound rat ACE has a marginally greater affinity (K_m 1 mM), but a substantially lower V_{max} (0.261 μmol/min/mg) for the hydrolysis of the same substrate at pH 8.3 (Yoshioka et al., 1987).

The principal member of the second group of carboxypeptidase enzymes, glutamate carboxypeptidase II (GC2) (EC 3.4.17.21) is a member of the peptidase M28 family that can rapidly hydrolyze oligopeptides with *C*-terminal acidic amino acid residues (The UniProt Consortium, 2014, Barinka et al., 2002), and those with *C*-terminal methionine (Barinka et al., 2002). It has an optimal pH of 6.5 with a folyl-γ-Glu-γ-[^{14}C]Glu substrate(Halsted et al., 1998).

Of the *C*-terminal exopeptidases, GC2 is the most abundant, comprising around 10 % of spectral counts identified in the BBMV murine proteome as BB peptidases. Angiotensin converting enzyme comprises 6 % while CPP was either not detected or fell below the reporting threshold , *i.e.* CPP contributed < 2 distinct protein sequences and < five spectra over the entire proteomic data set (McConnell et al., 2011).

Dipeptidases

A single BB enzyme is currently classified as a dipeptidase: DP1. It is classified by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology as a true dipeptidase as is indicated by the EC prefix numbers “3.4.13.-”, and is a BB member of the M19 family of peptidases (The UniProt Consortium, 2014, Kera et al., 1999). It accounts for 3 % of spectral counts identified as BB peptidase protein in the murine BBMV proteome (McConnell et al., 2011) and has a broad substrate specificity

(The UniProt Consortium, 2014) including leukotriene D4 and glutathione and its conjugates (The UniProt Consortium, 2014, Kozak and Tate, 1982). The authors have been unable to find any published data regarding the kinetics of the enzymatic activity of intestinal BB DP1, but renal papain solubilised and purified rat BB DP1 is reported to have a substrate affinity (K_m of 0.8mM) and a V_{max} of 1290 µmol/min/mg with an L-Ala-Gly substrate at pH 8 (Kozak and Tate, 1982). The optimal pH of porcine renal DP1 is 7.8 (Watanabe et al., 1996).

Endopeptidases

As noted earlier a few endopeptidases are also found in the BB (*e.g.* MEP and NEP). Meprin A subunit β hydrolyzes peptides, including azocasein, and insulin *β*-chains and has a preference for acidic residues at the P_1 and P'_1 positions (The UniProt Consortium, 2014, Bertenshaw et al., 2001). Five percent of spectral counts identified as BB peptidase protein in the murine BBMV proteome were identified as MEP (McConnell et al., 2011). The most prevalent BB endopeptidase is NEP. It is a single pass type II membrane endopeptidase of the M13 family of metalloproteases, which accounts for 10 % of spectral counts identified as BB peptidase protein in the murine BBMV proteome (McConnell et al., 2011). Neprilysin displays broad substrate specificity, preferentially cleaving oligopeptides between hydrophobic residues; hydrolyzing peptide bonds on the *N*-terminal side of hydrophobic amino acids such as leucine, tyrosine, phenylalanine, valine and tryptophan (Bunnett et al., 1988, The UniProt Consortium, 2014), and has a preference for phenylalanine or tyrosine at P'_1 (The UniProt Consortium, 2014). Porcine NEP has a pH optimum of 7 (Danielsen et al., 1980b). Assayed at pH 7.4, recombinant human NEP has a K_m for angiotensin I of 0.055 mM, a k_{cat} of 34.1s^{-1} and a k_{cat}/K_m of 6.2 x10^5 $M^{-1}.s^{-1}$ (Rice et al., 2004). Thus it shows a level of substrate affinity and catalytic

efficiency that is comparable to that of the hydrolysis of angiotensin I by human recombinant ACE K_m 0.019 mM, k_{cat} 3.5 s^{-1}, and k_{cat} /K_m of 1.8 x10^5 $M^{-1}.s^{-1}$(Rice et al., 2004).

The published work regarding the oligopeptidase consortium suggests that most have an optimal pH of around 7–8; hence, it is likely they may act at a different intestinal site from that of oligosaccharides, which have lower optimal pH of around 6. The kinetic activities of the peptidases suggest that they are catalytically efficient enzymes that, in general, have a high turnover rate. It appears their activity is constrained when the enzymes are bound to BBMV and solubilisation engenders greater activity. In this case BBMV may act as reservoirs of enzymes awaiting solubilisation by detergent (bile) or by pancreatic proteolysis as a means of efficient transport of enzymes through the mucin layer.

2.3.3 Lipolytic consortium

The actions of lipolytic BB enzymes, like those of all lipolytic enzymes, are complicated by the fact that they operate at the oil-water interface. Hence, they can be influenced by the actions of agents that influence the surface energies at the interface, *e.g.* emulsifying agents, or partition across the interface causing steric hindrance, *e*.g. amphoteric proteins. The most common BB lipases are discussed below.

Phospholipase B1

Phospholipase B1 is a single pass type I integral membrane protein of the GDSL lipolytic enzyme family/phospholipase B1 subfamily (The UniProt Consortium, 2014), and accounts for a quarter of spectral counts identified as BB lipolytic enzymes in the murine BBMV proteome (McConnell et al., 2011). It has two active sites, namely a PLA2 (EC 3.1.1.4) and a lysophospholipase (EC 3.1.1.5) site (The UniProt Consortium, 2014, Tojo

et al., 1998). The PLA2 site preferentially hydrolyzes diacylphospholipids and diacyglycerol at the sn-2 position, but also hydrolyzes triacylglycerol with no positional specificity (The UniProt Consortium, 2014). The PLA2 site also hydrolyzes phosphatidylethanolamine, choline plasmalogen and phosphatides (IUBMB, 2013). The lysophospholipase site hydrolyzes 2-lysophosphatidylcholine to glycerophosphocholine, releasing a carboxylate (IUBMB, 2013). The PLB1 complex can also hydrolyze retinyl palmitate (Rigtrup et al., 1994). The activity of PLB1 is augmented by bile (Rigtrup et al., 1994), which facilitates the location of the enzyme on the oil-water interface (Maldonado-Valderrama et al., 2011). The optimal pH of PLB1 is reported to lie between 8 and 9 with a 1-palmitoyl-*sn*-glycero-3-phosphocholine substrate (Tojo et al., 1998). The hydrolysis of phosphatidylcholine by papain solubilised and purified rat PLB1 has a K_m of 0.0113 mM and a V_{max} of 13.9 μmol/min/mg (1 % taurocholate pH 8) (Rigtrup et al., 1994).

Alkaline sphingomyelinase and neutral ceramidase

Other lipolytic BBMV enzymes hydrolyze dietary sphingolipids. Brush border AS and NC (Vesper et al., 1999), together account for two thirds of spectral counts identified as BB lipolytic enzymes in the murine BBMV proteome (McConnell et al., 2011). Alkaline sphingomyelinase is responsible for the hydrolysis of sphingomyelin to ceramide and phosphocholine, while NC hydrolyzes ceramides to sphingosine and fatty acids (The UniProt Consortium, 2014). The combined action of AS and NC results in the hydrolysis of dietary sphingolipids (Vesper et al., 1999) that bypass pancreatic digestion (Beck, 1973, Vesper et al., 1999). There is little comparable kinetic data available for these enzymes. Triton X-100 solubilised and purified NC obtained from human ileostomy effluent has a K_m of 0.0132 mM and a V_{max} of 0.807 μmol/min/mg with the substrate

octanoyl–sphingosine in the presence of 10 mM sodium taurocholate (NTC), pH 7 (Ohlsson et al., 2007). In contrast, Triton X-100 solubilised and purified rat NC, collected by *ex vivo* flushing of the small intestine with taurodeoxycholate, had a K_m of 0.074 mM and a V_{max} of 160 μmol/min/mg with a [^{14}C] octanoyl–sphingosine substrate (pH 7, 10 mM NTC) (Olsson et al., 2004). Neutral ceramidase has an optimal pH between 7 and 7.5 (Ohlsson et al., 2007, Lundgren et al., 2001) and is bile salt dependent (Lundgren et al., 2001). The large difference in V_{max} suggests that the rat NC is more efficient in hydrolysing sphingolipids than human NC. However, it is possible that human NS, having been collected from ileostomy effluent, may have been degraded during intestinal transit.

2.4 Conclusions

Our current understanding of the physical and chemical dynamics of mammalian intestinal BB enzymes is incomplete. While we have a broad outline of the processes of the genetic regulation and synthesis of BB enzymes within the enterocyte, many questions remain about their transit and incorporation into the microvillus membrane, the shedding of the membrane bound assemblage *in situ* as BBMV, and the shedding of soluble versions of BB enzymes from the enterocyte and BBMV. In particular, the roles of the BBMV-bound enzymes and their associated microenvironments, the extent of peripheral containment of BBMV and the significance of the differences in the kinetic profiles of cytosolic, BBMV-bound and soluble extracellular enzymes all remain unclear.

This review has shown that it is possible to isolate a preparation of catalytically active BB enzymes, attached to a membrane vesicle. The BBMV provides a scaffold that allows a consortium of enzymes to coexist in physiologically relevant concentrations. Hence, this thesis will investigate the premise that a BB digestive phase may be prepared characterized and used as an adjunct to typical luminal methods of in vitro digestion, to allow a more complete and valid representation *in vivo* digestion than can be obtained by

methods based on buccal/gastric and pancreatic enzyme combinations without a BB phase.

3 Chapter 3. Methods. Brush border preparation and assay

The previous chapter demonstrates that a consortium of BB enzymes are secreted, attached to BBMV, and can be purified from small intestinal tissue for characterisation *ex vivo*. The aim is that the BBMV fraction can then be used as an adjunct to *in vitro* digestion methods, where information on the terminal pre-absorptive enzymatic digestion products are of interest, as in this thesis. This chapter progresses towards that aim by outlining the processes and methodology involved in the isolation and characterisation of BB enzymes from rat mucosal tissue, and details the buffers and pH conditions used in assays and the BB *in vitro* digestion method.

3.1 Reagents

Where possible all reagents were of premium or analytical grade. The reagents and their catalogue numbers were; Bile extract (porcine) (B-8631) pancreatin (porcine) (P7545), Glucose standard (G6918), 4-Nitroaniline (N2128), Mannitol (M4125), L-*N*-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (S8511), L-leucine-*p*-nitroanilide (L9125), Isomaltotriose (I0381), Maleic acid (63180) Maltose (potato) (M-5885), phosphoramidon (R7385), magnesium chloride (M8266) calcium chloride (C1016), manganese chloride (M8054), β-Lactose (L3750), 4-nitrophenol (97660), and *p*-nitrophenyl-phosphate (N2770), sodium phosphate monobasic monohydrate (S-9638), sodium phosphate dibasic anhydrous (S-9763) were obtained from Sigma-Aldrich. Sucrose (530) was purchased from Ajax chemicals, GOPOD (K-GLUC), pullulan (P-PULLN) from Megazyme International, and Glycine (104201.1000) and zinc acetate (108802.025) from Merck. Sodium chloride (VWRC27800.360), Sodium hydroxide from BDH (VWRC28244.295).

LC-MS grade acetonitrile was from Fischer Scientific, methanol (ChromAR) was from Mallinckrodt Chemicals, and ethanol (95 %) was from LabServ.

3.2 Animals

3.2.1 Animal models

Ideally human BB enzymes would be used to model human digestion *in vitro*. However, ethical issues, and access to human small intestinal samples, preclude these from being used in the development of a BB digestive phase. Although pigs are physiologically suitable for modelling human digestion due to porcine diet and physiology (Nielsen et al., 2014) it was not logistically feasible to use them in the current developmental research. The model animal used for experimental purposes was the rat. The transit time of digesta from ingestion to the terminal ileum for both rats and humans is 3-4 hours, and protein digestion in both mammals is comparable (Deglaire and Moughan, 2012).

3.2.2 Sprague Dawley rats (*Rattus norvegicus*)

Sprague Dawley rats were bred and raised at the Food Evaluation Unit, Plant & Food Research, Palmerston North, in a room maintained at a temperature of 22 ± 1^{o}C; humidity was 60 ± 5 %; the air was exchanged 12 times/hour, and there was a 12 hour light/dark cycle. As the rat breeding facility is part of Plant and Food Research, the husbandry, diet and the mucosal sampling (AEC numbers 12356, 12687, 13164) of all animals could be monitored.

Male rats were chosen for this study due to anatomical differences in the length of the small intestine between genders. The average length of the rat small intestine differs by 10 cm between a male and female rat at 12 weeks, 116 vs. 106 cm respectively (Blair et

al., 1963). These authors also showed there were significant differences in sucrase activities with age (Blair et al., 1963).

From weaning to 8 weeks eight Sprague Dawley rats were fed Specialty Feeds meat free rat and mouse diet. From 8 weeks rats were placed on an *ad libitum* (≤35 g/day) standardised pelleted diet (AIN93G) (Reeves et al., 1993), and moved into hanging cages (with no wood shavings) to avoid any differences in enzyme activities caused by the consumption of wood shavings. Rats were given two weeks to acclimatise to new conditions, and at 10 weeks were ready for tissue harvest. Water was available *ad libitum*, but food was withdrawn 16 hours before euthanasia. Fasting prior to euthanasia was undertaken to minimise fluctuations in the concentrations and specific activities of BBMV enzymes in response to food intake (Tanaka et al., 2008, Goda., 2000, Ferraris et al., 1992). Primary work indicated that there were significant inter-animal differences in enzyme activities, which meant that the BBMV fractions from 8 rats were pooled for all trials (appendix 4).

Rats were euthanased by carbon dioxide inhalation, and tissue samples were taken *post-mortem*. The small intestine (duodenum, jejunum and ileum) was removed by dissection, flushed with ice cold sterile 0.9 % sodium chloride, and placed on an ice cooled metal tray. Small intestinal mucosae were then ready for sampling.

3.3 Isolation and purification of brush border membrane vesicles (BBMV)

The isolation and purification of BBMV involves multiple steps. The first is the isolation and homogenisation of mammalian small intestinal mucosa. The second step is the addition of a divalent cation to the homogenate to precipitate baso-lateral and intracellular membranes, and thirdly the treated homogenate is subjected to differential centrifugation

to isolate BBMV. Factors affecting these processes, and their relevance to the selection of method used in this thesis are detailed below.

3.3.1 Factors affecting the preparation of BBMV

The initial step in the purification of BBMV is to isolate mammalian small intestinal tissue. The mucosae are then removed either by micro-dissection from the underlying *muscularis mucosae* (Schmitz et al., 1973) or scraped from the surface of the small intestine (Kessler et al., 1978). As the literature has suggested that no significant difference between the methods was observed, in the resulting BBMV fraction, it was decided that we would not use the BB dissection method as it would be time consuming, and therefore not appropriate for purifying large amounts of BBMV. Following isolation, mucosal samples are added to buffer and homogenised. The homogenised mucosal samples then undergo divalent cation precipitation, prior to centrifugation.

Divalent cation precipitation

The addition of divalent cations, such as calcium or magnesium, is the most common way of removing contaminating membranes from mucosal homogenates. The choice of cation depends on what is being investigated. Although there is no apparent difference in the yield of BBMV produced from calcium or magnesium precipitation (Schmitz et al., 1973, Kessler et al., 1978, Booth and Kenny, 1974) some studies suggest magnesium precipitation may result in a less pure BBMV fraction (Kessler et al., 1978). Other authors recommend that calcium precipitation should be avoided if the aim is to study BBMV membrane lipids as calcium can increase the activity of BB phospholipid hydrolysing enzymes, such as PLA2, which may alter the composition of membrane lipids (Subbaiah and Ganguly, 1970). The alteration of membrane lipids may affect the fidelity of the

BBMV, and disperse component membrane lipids. Altering enzyme stability (White and Wimley, 1998), and orientation thus affecting enzyme activities. Conversely calcium is thought to be important for maintaining the structural integrity of the BB (Miller and Crane, 1961b, Millington et al., 1966) so the presence of some calcium may be advantageous. Because oligosaccharidase and peptidase activities rather than lipid hydrolysis was the important focus of this thesis it was decided that Ca^{2+} need not be avoided in BBMV. However, micrographs of the BBMV fraction indicate that BBMV produced displayed normal morphology and orientation (section 3.4.1), which was in keeping with previous reports, and suggested there was no membrane dispersion.

The homogenate then undergoes differential centrifugation resulting in a preparation that is basically free of mitochondrial cytosolic, microsomal, nucleic contaminants and baso-lateral membrane, and is enriched in BB marker enzymes (Kessler et al., 1978).

Purification methods

The most common method used for the isolation of BBMV is differential centrifugation. Further isolation steps, to improve the homogeneity of the BBMV fraction, include chromatography (Ohsawa et al., 1979), and more commonly, density gradient centrifugation (DGC) (Schmitz et al., 1973). Density gradient centrifugation involves applying the BBMV fraction from the differential centrifugation step onto a sorbitol, sucrose or glycerol gradient. In the case of a sucrose gradient, fraction 3 is highly enriched with BBMV (McConnell et al., 2009). Fraction 3 includes the 16-25 % interface and has no detectible actin filaments suggesting that these BBMV do not result from BB fragmentation (McConnell et al., 2009). If a density gradient centrifugation step was included this would result in an additional 18 hours of centrifugation (McConnell et al., 2009). Further, high concentrations of glycerol can inhibit oligosaccharidases, and also

have the potential to interfere with enzyme activities (Eichholz and Crane, 1965). Hence, the compound used to form the density gradient needs to be carefully chosen so as not to inhibit BB enzyme activity. Since such purification techniques result in only a small increase in purity and enzyme marker enrichment (Kessler et al., 1978) further purification steps were not used.

Where the purification of individual enzymes is required immunoelectrophoresis and immunochromatography can be used to purify individual enzymes to a homogenous fraction (Semenza, 1986). However, this method involves dissociating enzymes from BBMV, which may alter the activity of the enzyme (Hooton et al., 2015), and the development of an *in vitro* BB system will require the full complement of BB enzymes, attached to the BBMV, in order to maintain the fidelity of the BB phase. Hence, it was decided that such purification steps were not warranted.

Potential additions to the purification procedure

Some methods incorporate procedures, other than those described above, to the process of BB membrane purification. For example Tris can be used to disrupt and fragment BB membranes (Eichholz and Crane, 1965, Schmitz et al., 1973) and is used as a buffer (Farooq et al., 2004). Tris is known to inhibit oligosaccharidases (Dahlqvist, 1961) so was not used in the chosen purification process, as inhibiting the activities of any BB enzymes would be deleterious. Thiocyanate salts are often used to separate the BB from cytoskeletal components (Hopfer et al., 1983). However, thiocyanate salts can interfere with hydrophobic interactions (Hopfer et al., 1983) so have the potential to disrupt the tertiary structure of BB enzymes, and the fidelity of BBMV. Thiocyanate is also highly toxic to humans, so for these reasons was not used.

Another compound used during purification is ethyl-diamine-tetra-acetic acid (EDTA). This is used mainly to chelate divalent cations (Millington et al., 1966), such as calcium and magnesium. Again, calcium is thought to be important for maintaining the structural integrity of the BB (Miller and Crane, 1961b, Millington et al., 1966). Neither thiocyanate nor EDTA were incorporated into the purification procedure.

Centrifugation speed

The speed of the centrifugation steps used in differential centrifugation differs from publication to publication. In general there is a slow spin of 3,000-5,000 x *g* to remove cellular debris, followed by 1-5 high speed centrifugation steps with speeds as low as 12,000, but more often between 27,000 and 48,000 x *g* to isolate BBMV (Boutrou et al., 2008, Kessler et al., 1978, Booth and Kenny, 1974, Schmitz et al., 1973, Hira et al., 2001). The differences in rotor speed were likely to be related to the availability of rotors.

For the purposes of the work described in this thesis the most commonly used speeds were chosen, *i.e.* 3,000 x *g* and 27,000 x *g* respectively. It was important that the centrifugation speed was not too high as centrifugal speeds of 100,000 x *g* would result in the pelleting of soluble enzymes with the BBMV (in this case cytosolic enzymes). A literature search did not reveal why particular centrifugation speeds and times were used. However, published works have shown that the final pellet fractions are enriched with BBMV enzyme markers, while other fractions are enriched with enzyme markers from other cellular components, *i.e.* nucleic (pellet 1), mitochondrial (pellet 1), cytosolic (supernatant II) and basolateral membranes and organelles (pellet 1) (Schmitz et al., 1973, Kessler et al., 1978, Ohsawa et al., 1979). Further, microscopic analysis of BBMV fraction shows the presence of large quantities of BBMV (Kessler et al., 1978). Similar

methods show no contamination with membranes from other organelles (Schmitz et al., 1973).

3.3.2 Method for the isolation and purification of BBMV

As the aforementioned discussion has shown, there are a many factors to consider when deciding which purification method suits particular research requirements. For this project Ca^{2+} precipitation and differential centrifugation were used as the purification process was quick, simple, and based on previously validated methods ((Boutrou et al., 2008, Kessler et al., 1978, Sakuma et al., 2009), figure 4).

Following dissection, the small intestine was opened longitudinally with the mucosal side up. The luminal surface of the mucosa was scraped from the underlying tissue using a flat metal spatula angled at 45º. The mucosal scrapings of 8 rats were pooled for preparation of the BBMV. Ice cold buffer 1 was added to the mucosal scrapings at 30 mls of buffer 1 per gram of mucosal scrapings. Buffers differed depending on the objectives of the experiments. All buffers used in the preparation of the BBMV fraction are described in section 3.3.3.

The suspended mucosal scrapings were homogenised using an Omni THq homogeniser at 35 rpm for 30 seconds. Calcium chloride (1 M) was added to the homogentate, to a final concentration of 10 mM, and the homogenate placed in an ice bath for 30 minutes. Calcium precipitation was used to isolate BBMV and the soluble fraction from other cellular components by causing the aggregation of microsomal vesicles, lysosomes, mitrochondria and basolateral membrane (Schmitz et al., 1973). The homogenate was centrifuged at 3,000 x *g* in a Sorvall RC5B plus centrifuge (Sorvall SLA 1500 rotor) for 15 minutes at 4 ºC, causing the aggregated membrane and other cellular debris to pellet. The pellet was discarded and the supernatant (supernatant 1) was centrifuged at 27,000 x

g in a Sorvall RC5B plus centrifuge (Piramoon F21 rotor) for 30 minutes at 4ºC. The resulting supernatant (supernatant 2) was kept for further analysis (Chapter 6 and 7) or more often discarded, and the precipitate was dispersed in ice cold suspension buffer (buffer 2). The re-suspended pellet was then centrifuged a second time at 27,000 x *g* and again the supernatant was removed and the pellet re-suspended in ice cold suspension buffer. The resulting fraction was termed the BBMV extract. Further extraction was undertaken to obtain solubilised BB enzymes in some experiments, section 4.4. All extracts were diluted in suspension buffer (buffer 2) prior to use.

Potential contamination

The purification process involved multiple handling steps all of which had the potential to introduce contaminating micro-organisms from the environment or large intestine. Local segmental contractions in the distal small and proximal large intestine cause the bidirectional flow of digesta between the small and large intestine (Janssen et al., 2007). This results in distal ileal contents being mixed with large intestinal contents, including enteric microbiota, which may contaminate the BBMV fraction. There was also the need to store purified BBMV for later use. To ensure no bacterial activity occurred the bacteriostatic agent sodium azide (NaA) was used at a concentration of 0.05 %. Sigma product information suggests that a NaA concentration in the range of 0.02-0.1 % is effective at inhibiting bacterial activity. However, literature suggests that although 0.01-0.03 % NaA acts as a bacteriostatic for gram negative bacteria, 0.03 % is not enough to inhibit the gram positive bacteria: *Streptococci*, *Pneumococci* and *Lactobacilli (Lichstein and Soule, 1944).* Sodium azide was added at 0.05 %, a concentration that appears effective in inhibiting bacterial activity without altering enzymatic activities to a large

degree. There was no evidence of bacterial or fungal growth following the addition of NaA (results not shown).

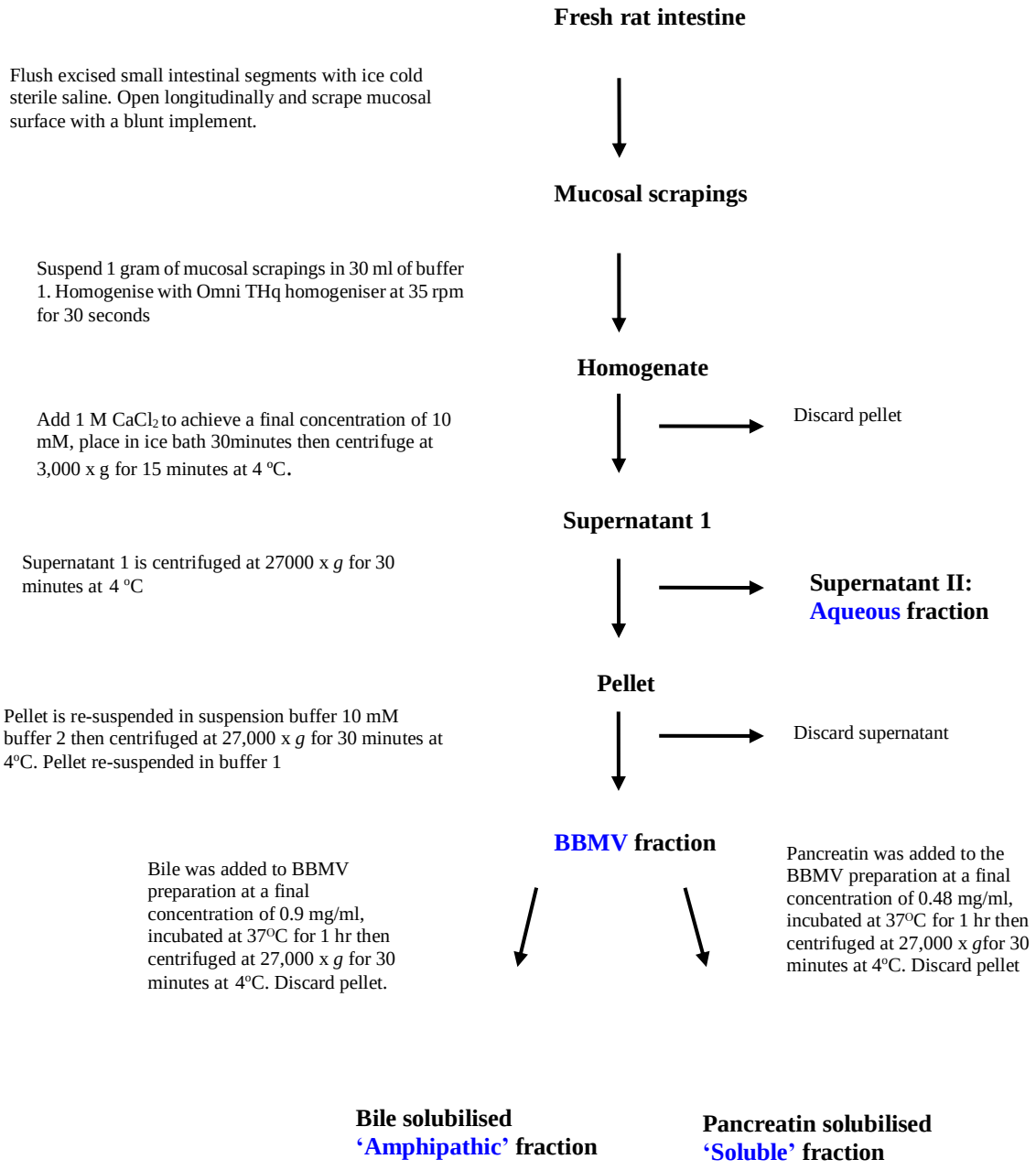

Figure 4. Overview of calcium precipitation and differential centrifugation methodology and the subsequent solubilisation of BBMV enzyme

3.3.3 Buffers

This section describes the formulation of specific buffers required for the preparation and assay of BBMV fractions, and enzymes with specific requirements. The buffers described in this section differ depending on the objectives of the work. An explanation for the chosen pH concentrations of the buffers chosen will precede the description of these buffers used in one or more chapters.

3.3.3.1 Buffers for chapter's 5, 6 and 8

The primary aim of chapter 5 was to determine the enzyme kinetics of BB enzymes from the oligosaccharidase and oligopeptidase consortium under physiologically relevant conditions and pH's using the rationale outlined in section 4.3.

The primary aim of chapter 6 was to determine whether BB enzyme activities were affected by exposure to physiologically relevant concentrations and volumes of biliopancreatic secretions. The exposure assays were undertaken using the *in vitro* rationale outlined in section 4.3.

The primary aim of chapter 8 was to validate the use of the *in vitro* method based on the rationale outlined in section 4.3, using polyphenolic compounds as substrates.

The criteria for the selection of buffers were:

1. To be physiological relevant.
2. To accommodate for the pH sensitivities of the majority of BB enzymes.
3. To be compatible with pH changes along the apical surface of the small intestine.
4. To be compatible with the addition of divalent cations.

The phosphate buffering system was chosen as it is an important biological buffering system in the mammalian body, and buffers in the pH ranges of BB enzymes. The pH optimums of enzymes of the main oligopeptidase and oligosaccharidase enzymes are shown in figure 5.

The oligosaccharidase enzymes cluster around pH 6 , which is in keeping with the pH of the peri-apical space of the proximal jejunum (pH 6.1) (Lucas, 1983) and the pH optimums of SI: 5.9 (50 mM sodium) (Kolínská and Kraml, 1972), MGAM: 6.5 (Sorensen et al., 1982), and LPH 5.9 (Skovbjerg et al., 1982). In contrast the pH optimums of the oligopeptidase consortium were clustered around pH 8, which is comparable with the pH optimums of the primary oligopeptidases from each classification *i.e.* *N*-terminal peptidases: APN, 7.5 (Caporale and Troncone, 1988), *C*-terminal peptidases: ACE, 8.4 (Yoshioka et al., 1987); endopeptidases: NEP, 7 (Danielsen et al., 1980b); and dipeptidases: DPI, 8 (Kozak and Tate, 1982). Further, a pH of 8 is in keeping with the pH of 7.3 the peri-apical ileum (Lucas, 1983).

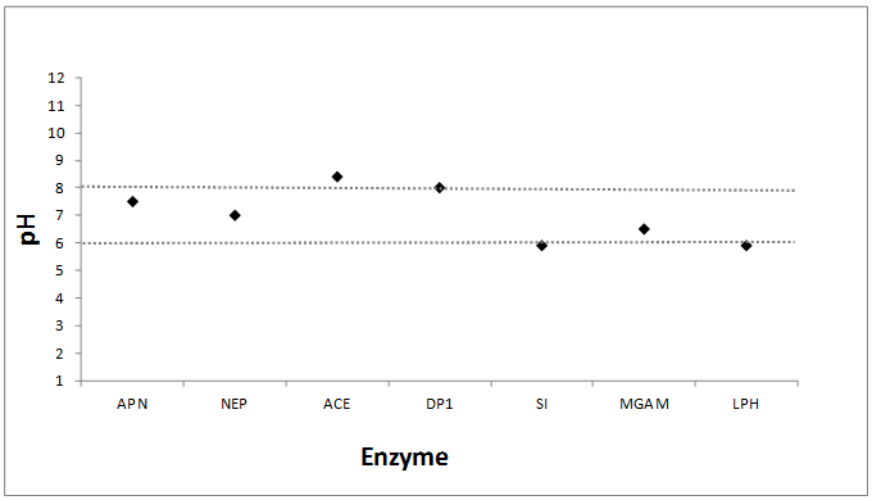

Figure 5. The pH optimums of a selection of brush border digestive enzymes

pH optimums and authors: APN: 7.5 (Caporale and Troncone, 1988), ACE: 8.4 (Yoshioka et al., 1987); NEP: 7 (Danielsen et al., 1980b) ; DPI: 8 (Kozak and Tate, 1982); SI:5.9 (Kolínská and Kraml, 1972); MGAM: 6.5 (Sorensen et al., 1982); LPH 5.9 (Skovbjerg et al., 1982). The dotted lines at pH 6 and 8 indicate the peri-apical pH of the proximal jejunum and distal ileum respectively(Lucas, 1983)

Phosphate buffer: pH 6

For the isolation of BBMV, and for assaying the oligosaccharidase enzymes, the buffers used were pH 6. Buffer 1 was a 2 mM phosphate buffer with divalent cations (table 2) and 50 mM of mannitol, pH 6. This buffer was hypotonic to facilitate the lysis of enterocytes and organelles during the purification process. Buffer 2 was a 10 mM phosphate buffer with divalent cations and 50 mM of mannitol, pH 6. All reagents were prepared, and enzymes assayed, in buffer 2.

Phosphate buffer: pH 8

For the isolation of the BBMV fraction and for assaying the peptidase enzymes the buffers used were pH 8. A hypotonic 2 mM phosphate buffer (50 mM mannitol and divalent cations, pH 8) was used for the isolation of BBMV (buffer 1) and the pellet was reconstituted in 10 mM phosphate buffer (50 mM mannitol and divalent cations, pH 8,

buffer 2). This buffer was monitored for signs of precipitation as the addition of divalent cations increased the chance of precipitation at pH 8.

Table 2. The buffers used in the preparation and assay of BBMV enzymes in chapter 5 and 8

Buffer	Enzymes	Buffer*#	Concentration (mM)	pH
1	Oligosaccharidases	Phosphate buffer	2	6
2	Oligosaccharidases	Phosphate buffer	10	6
1	Peptidases	Phosphate buffer	2	8
2	Peptidases	Phosphate buffer	10	8

*All buffers contained 50 mM mannitol; #divalent cations added

3.3.3.1.1 Additions to buffers 1 and 2

Mannitol

All buffers contained mannitol at a concentration of 50 mM as it is reported to stabilise the tertiary structure of enzymes by reinforcing the hydrophobic interactions of non-polar amino acids (Iyer and Ananthanarayan, 2008). While also helping to maintain the hydration shell that surrounds enzymes (Kaushik and Bhat, 1998, Schein, 1990).

Cofactors: the addition of divalent cations

Divalent cations were added to all buffers in concentrations that allow BB enzymes with known cofactors to function maximally (The UniProt Consortium, 2015). For example, zinc is an essential cofactor for APN and NEP (The UniProt Consortium, 2014). The four cations were added at concentrations that mimicked the distribution of these elements within mammalian cells (table 3), and were included at levels below those that are deemed toxic (Williams and Fraústo da Silva, 2000). The addition of these cations may have the added benefit of stabilising the tertiary structure of enzymes (Iyer and Ananthanarayan, 2008), and so may promote enzyme hydrolysis. The buffers were autoclaved prior to the

aseptic addition of the divalent cations in order to minimise the chance of precipitates forming.

Table 3. Concentrations of the cations added to buffers 1 and 2 (pH 6 and 8)

Ionic species	Concentration
Zinc	0.01 pM
Magnesium	1 mM
Manganese	1 µM
Calcium	1 µM

Catering for the lipolytic consortium

Although BBMV incorporate a consortium of lipolytic enzymes (section 2.3.3), most BB lipolytic enzymes (lipases and phospholipases) do not cleave substrates at molecular sites that are distinct from those cleaved by enzymes of gastric and pancreatic origin (Bauer et al., 2005, Gassama-Diagne et al., 1989, IUBMB, 2013). Although sphingolipid hydrolysing BB enzymes have unique activities (The UniProt Consortium, 2015, Vesper et al., 1999) the estimated average intake is 0.3–0.4 grams per day (Vesper et al., 1999). It was decided that accommodating the requirements of these enzymes was not required. Neutral ceramidase and AS have pH optimums of 6 (Olsson et al., 2004) and 9 (Nilsson and Duan, 1999) respectively; so at pH 6 NC will function optimally, but at pH 8 AS will have approximately 10 % of the activity seen at its pH optimum of pH 9 (Nilsson and Duan, 1999). If researchers are interested in adapting this system to study sphingolipid hydrolysis then pH adjustment could be made latterly.

3.3.3.2 Chapter 6 buffers continued

The objectives in chapter 6 were twofold. Firstly, to determine whether there were temporal changes in BBMV-bound and aqueous BB enzyme activities when exposed to bile ± pancreatin using the *in vitro* digestion method found in section 4.2.

The second objective was to assess the activities of BBMV enzymes exposed to bile ± pancreatin at pH values chosen to replicate conditions of the small intestinal BB in an *in vitro* digestion. The buffers chosen for objective 2 are described in section 3.3.3.1.

To ensure the optimal activities of BB maltolytic enzymes the BBMV and aqueous BB fractions were prepared and assayed in buffers of pH 6.8 (table 4) in order to reflect the pH optimums of maltase and sucrase of 6.5 (Sorensen et al., 1982) and 6.7 (Kolínská and Kraml, 1972) respectively. The latter representing the pH optimum of sucrase in the absence of sodium (Kolínská and Kraml, 1972). These pH values are in keeping with range of pH values seen in the small intestine, *i.e.* 5.7-7.7 (Ekmekcioglu, 2002).

Two buffers were used in the preparation of the enzyme fractions buffer 1 was a 2 mM sodium hydrogen maleate buffer with 50mM mannitol, pH 6.8 (Dawson et al., 1986). The BBMV pellet was reconstituted in a 10 mM sodium hydrogen maleate suspension buffer with 50 mM mannitol, pH 6.8 (buffer 2) (Dawson et al., 1986). All reagents used in the assay of maltolytic activities were prepared in buffer 2.

The pH of the buffers used to prepare BBMV, and to assay ALP were of pH 9.4. This pH was chosen to reflect the pH optimum of the rat (pH 9) and human ALP (pH 9.5) (Bitar and Reinhold, 1972). The high pH optimum of ALP is thought to be due to ALPs role in mediating surface pH and bicarbonate secretion (Lalles, 2010). For the isolation of the BBMV fraction buffer 1 was a 2 mM glycine/NaOH buffer with 50 mM mannitol, pH 9.4, and the suspension buffer was 10 mM glycine/NaOH buffer with 50 mM mannitol, pH 9.4 (buffer 2) (Dawson et al., 1986). All reagents used in the assay of ALP activities were prepared in buffer 2.

Table 4. Buffers used in the preparation of BBMV and the temporal assay of enzymes

Buffer	Enzyme	Buffer*	Concentration (mM)	pH
1	Maltase	NH-maleate	2	6.8
2	Maltase	NH-maleate	10	6.8
1	ALP	Glycine	2	9.4
2	ALP	Glycine	10	9.4

*All buffers contained 50 mM mannitol

3.3.3.3 Chapter 7 buffers

The primary aim of chapter 7 was to determine whether BBMV-bound and solubilised BB enzymes were stable over a range of pH values. Each buffer was chosen for their capacity to buffer solutions over a wide range of pH's, which enabled the activities at various pH values to be assessed without the confounding influence of different buffer types.

Two buffers were used in the isolation of the BB enzyme fractions (table 5). The pH of the buffer depended on the enzymes being assayed. For the oligosaccharidases (maltase, sucrase and lactase) buffer 1 was a hypotonic 2 mM citric acid/phosphate buffer (Dawson et al., 1986) with 50 mM mannitol (pH 5.6), and buffer 2 was a 10 mM citric acid/phosphate buffer (Dawson et al., 1986) with 50 mM mannitol (pH 5.6). Fractions were then diluted from their original concentration of 33.3 mg/ml to 5 mg/ml with buffer of the appropriate pH (3.6 – 7.6). The pH was checked, and adjusted, aseptically prior to use using an ISFETCOM S2K922 micropH meter (Total Lab Systems Ltd). All reagents used in the assay of the oligosaccharidases were prepared in buffer 2.

For ALP buffer 1 consisted of a 2 mM Glycine/NaOH buffer (Dawson et al., 1986) with 50 mM mannitol (pH 9.6) and buffer 2 was a 10 mM glycine/NaOH buffer (Dawson et al., 1986) with 50 mM mannitol (pH 9.6). Again aliquots of enzyme were diluted from

the original concentration of 33.3 mg/ml to 5 mg/ml with buffer (final concentration after admixture 2 mg/ml) of the appropriate pH (8.6 – 10.6) buffer and the pH checked aseptically prior to use.

Table 5. Buffers used in the preparation and assay of enzymes in chapter 7

Buffer	Enzyme	Buffer*	Concentration (mM)	pH
1	Oligosaccharidases	Citric acid/phosphate	2	5.6
2	Oligosaccharidases	Citric acid/phosphate	10	3.6, 4.6, 5.6, 6.6, 7.6
1	ALP	Glycine/NaOH	2	9.6
2	ALP	Glycine/NaOH	10	8.6, 9.6, 10.6

*All buffers contained 50 mM mannitol

3.4 Validation of the calcium precipitation and differential centrifugation method

It was important to know that BBMV obtained by calcium precipitation and differential centrifugation were orientated right side out to be sure that they were not an artefact of the purification process. Brush border membrane vesicles obtained via divalent cation precipitation with calcium or magnesium, and differential centrifugation or free flow electrophoresis were examined to assess membrane orientation (Haase et al., 1978). Immunological and electron microscopy freeze fracture methods show that 90 % of membrane vesicles are orientated in the same way as BB membrane *in situ* using these methods (Haase et al., 1978).

3.4.1 BBMV morphology

In order to ascertain whether the enzyme activity could be attributed to BB enzyme hydrolysis it was important to independently validate the method being used. Hence, a

sample was taken to the Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North, and with the help of Doug Hopcroft and Jianyu Chen transmission electron microscope (TEM) micrographs were taken of BBMV. The first technique used negative staining; a drop of the BBMV sample was dropped on to a clean parafilm surface, a carbon stabilised formvar grid was placed on the drop for 4 minutes, and the excess liquid was removed with the edge of a piece of filter paper, and the grid placed on to a drop of 2 % uranyl acetate in water, for 4 minutes. Extra fluid was removed and the grid allowed to dry before the sample was viewed. The TEM micrographs were resolved with a Phillips CM10 transmission electron microscope and digital images were obtained using an Olympus SIS Morada digital camera.

The second technique involved injecting a liquid BBMV sample into a 3 % agarose tube, which contained the sample throughout processing. The sample was fixed in 3 % glutaraldehyde, in 0.1 M Cacodylate buffer; was then buffer washed, and underwent a secondary fixation with 1 % Osmium Tetroxide in the same buffer, was again buffer washed, then dehydrated through a graded acetone series before infiltrating and embedding in Procure 812 epoxy resin. Sections from the cured resin block were cut with a diamond knife, stained with Uranyl acetate and lead stains, and viewed. The digital images are shown below (figures 6-11).

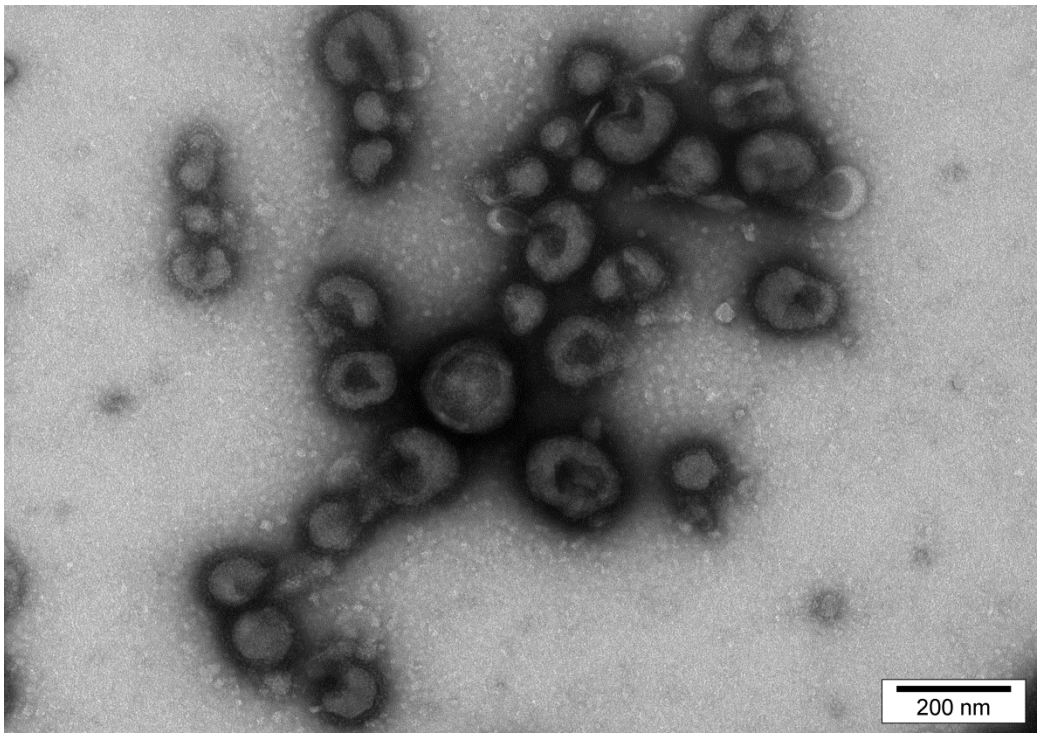

Figure 6. Transmission electron micrograph of BBMV following negative staining.

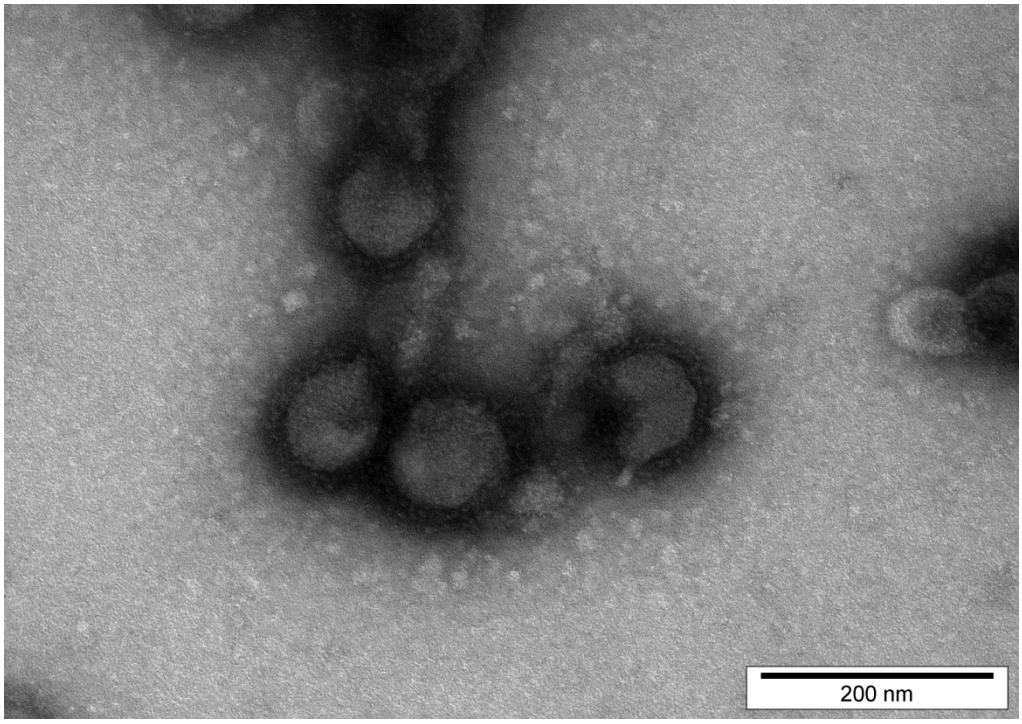

Figure 7. Transmission electron micrograph of BBMV following negative staining.

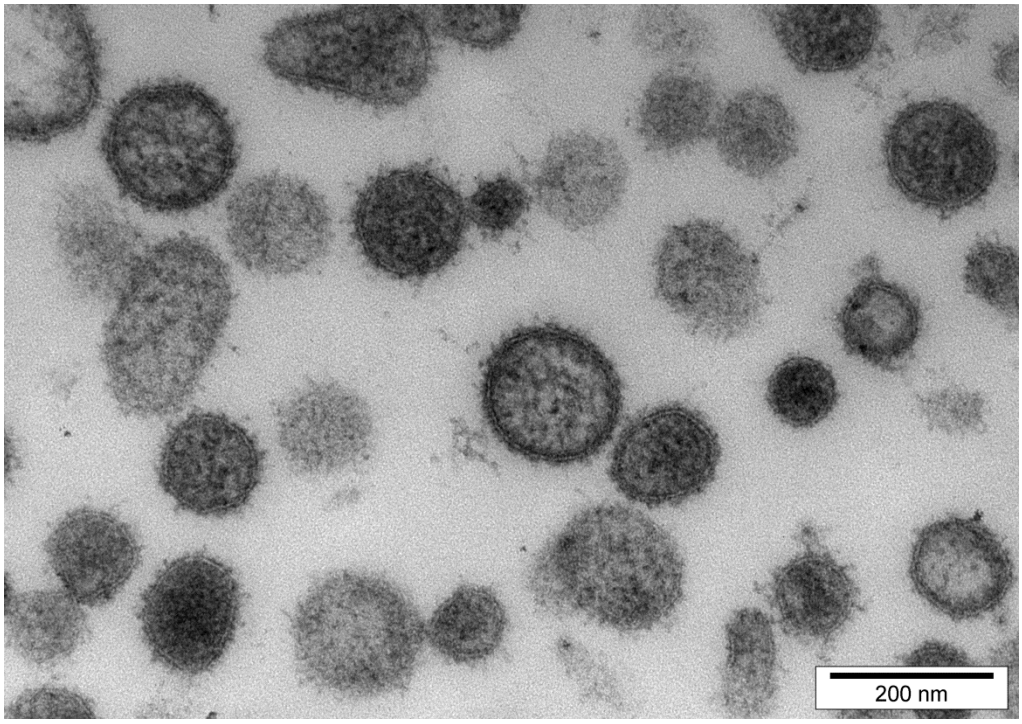

Figure 8. Transmission electron micrograph of BBMV imbedded in agarose

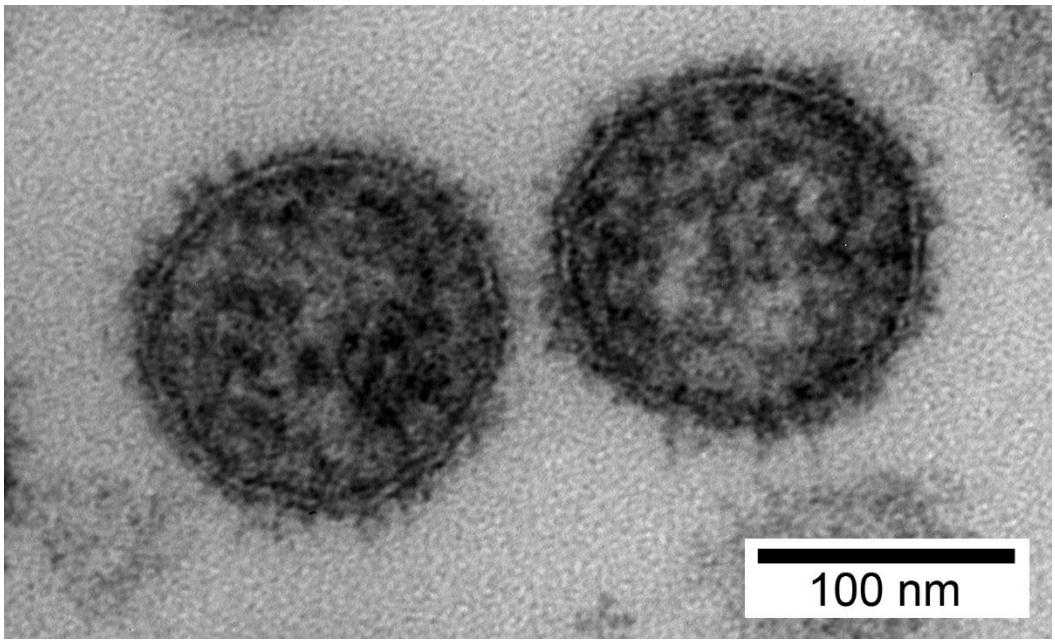

Figure 9. Transmission electron micrograph of BBMV that have undergone thin sectioning.

The micrographs, figures 6-9, show the presence of a multitude of bilayered vesicles consistent with BBMV (Kessler et al., 1978, Schmitz et al., 1973). Note, the knobbly looking outer layer covering the membrane bilayer of BBMV (figure 9). These structures are thought to be BB enzymes; they disappear from micrographs when enzymes are solubilised from the surface of BBMV by proteolysis (Maestracci, 1976).

3.4.2 Brush border membrane vesicle size

Another aspect of the validation was to estimate the size of BBMV in the BB preparation. The TEM micrograph (figure 10) was used to estimate the average BBMV particle size using Image J based software. The mean diameter of BBMV is reported to be 10 -125 nm (McConnell et al., 2009, Boffelli et al., 1997, Perevucnik et al., 1985). This is in keeping with the results shown here, which indicate the modal diameter ranges from 80 to 100 nm (figure 11).

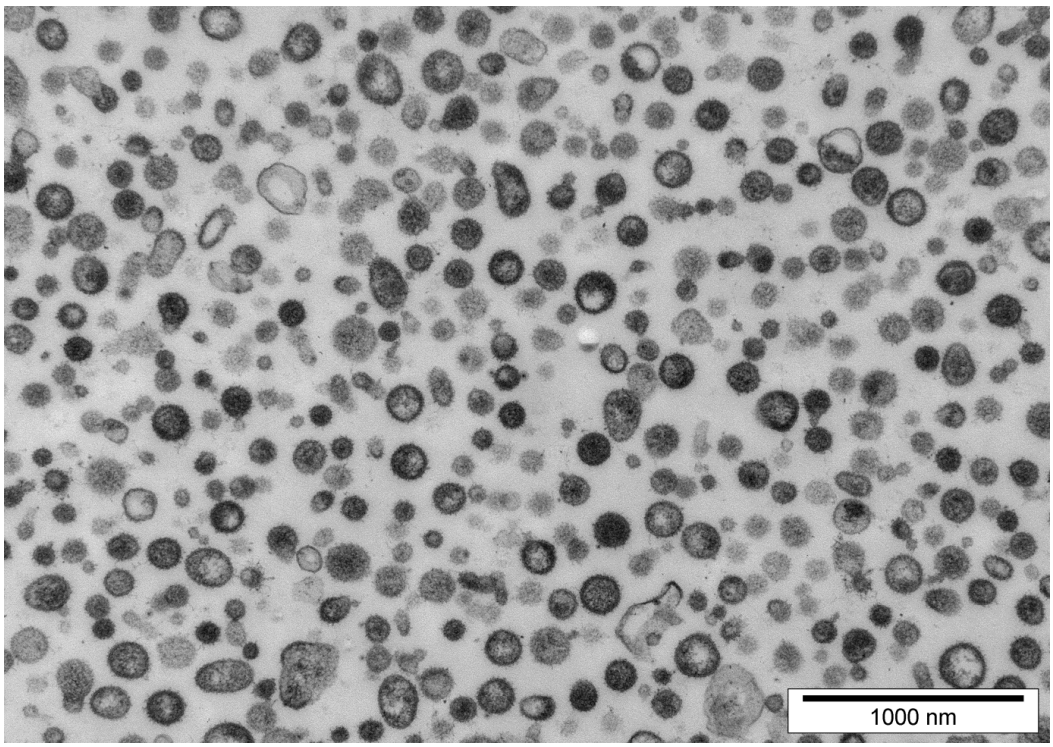

Figure 10. Transmission electron micrograph of BBMV that was used to estimate the size of BBMV using modified Image J software.

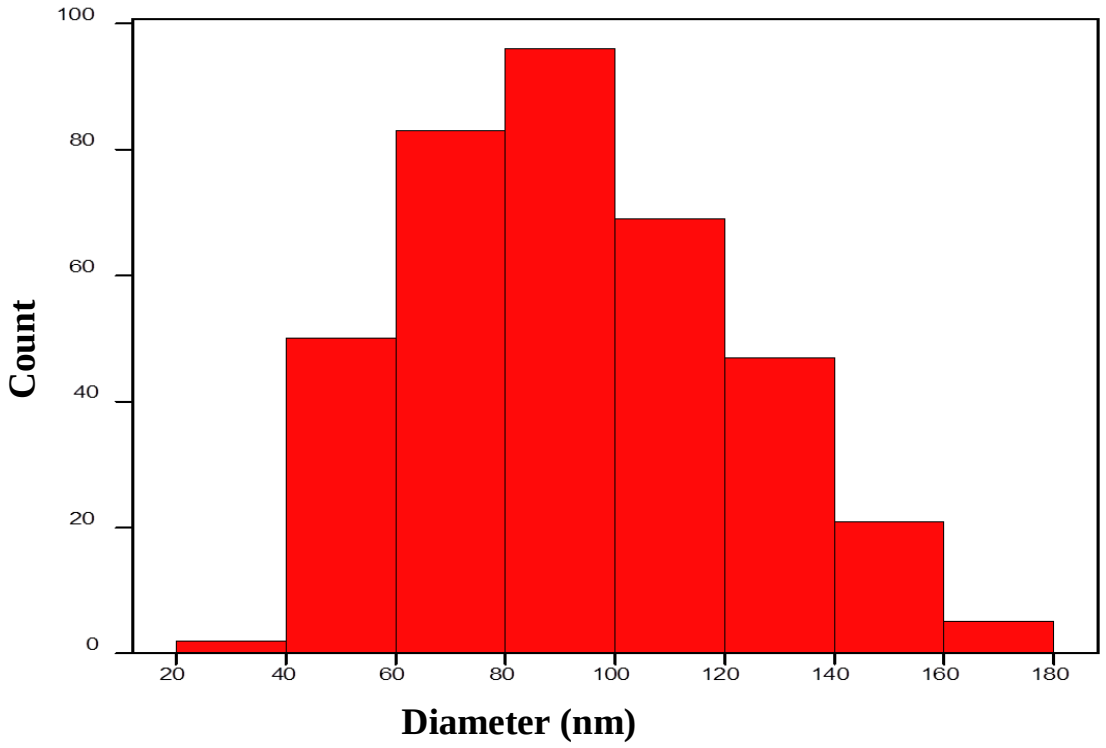

Figure 11. Graph showing the diameter of BBMV in figure 10.

Summary

The chosen method for calcium precipitation and differential centrifugation produced a functional BBMV fraction that was in keeping with previous work (Kessler et al., 1978).

3.5 Enzyme assays

The assay methods were chosen for their ability to be scaled to a 96 or 384 well plate. Absorbances were determined with a SpectroMax Plus spectrophotometer (Biostrategy, Auckland, NZ) with Softmax Pro analysis software 3.0 and Griener 96 (GR 655101) or 384 (GR7811010) well microplates. Any changes to the concentration of either the substrates or the BBMV fraction are detailed in the methods section of each chapter.

There are difficulties in the assay of the oligopeptidases as there is an overlap in the specificities of some BBMV aminopeptidases. Aminopeptidase N and DP1 have been shown to hydrolyse some of the same substrates (Kozak and Tate, 1982). The former displaying *N*-terminal exopeptidase (The UniProt Consortium, 2015) and dipeptidase activities, and the latter dipeptidase activity (Kozak and Tate, 1982). Dipeptidase 1 cannot hydrolyse Leu-*p*NA so this substrate was used to assess APN activities (Kozak and Tate, 1982).

Likewise there are difficulties in the assay of BBMV oligosaccharidases as individual substrates are hydrolysable by a consortium of enzymes. The oligosaccharidases (SI, MGAM and LPH) work in concert to cleave the glycosidic bonds of carbohydrate oligomers, namely α1,4 linkages (maltase, glucoamylase, sucrase and isomaltase), α1,6 linkages (isomaltase and to a lesser extent glucoamylase), α1,β2 linkages (sucrase), and β1,4 linkages (lactase and phlorizin-hydrolase) (Gray, 2000). Thus the substrates chosen for the characterisation of each enzyme should be specifically cleaved by that enzyme, but in some cases the specific linkage within a carbohydrate is able to be cleaved by a number of BBMV enzymes. Hence, in some cases we were restricted to assaying the

activities of a consortium of enzymes, *i.e.* maltase, glucoamylase, sucrase and isomaltase can all hydrolyse the α-1,4 bond of maltose. The specificity of each enzyme is detailed in section 2.2.2. The concentrations of substrates were, in some cases, limited by the solubility of substrates, *i.e.* Leu-*p*NA. Substrates are listed in table 6.

Table 6. Substrate and enzyme concentration for the kinetic investigation of BBMV- bound enzymes in chapter 4.

Enzyme	**Maltase**	**Sucrase**	**Lactase**	**Isomaltase**	**Glucoamylase**	**APN**	**NEP**
Substrate	Maltose	Sucrose	Lactose	Isomaltotriose	Pullulan	Leu-*p*NA	SAAPL-pNA
Highest concentration (mM)	10	250	375	25	7.5*	2.5	20
BB enzyme concentration (mg/ml)#	2	2	2	2	17	2	17

Key: * mg/ml; #: The final concentration of BBMV fraction in each assay. The concentration reflects the original concentration of mucosal scrapings in the homogenate from which BBMV fraction was isolated

3.5.1 Aminopeptidase N

Aminopeptidase N activities were measured by chromogenic assay (Fan et al., 2002, Bernkop-Schnürch et al., 1997, Haase et al., 1978) (figure 12). Leucine-*p*-Nitroanilide (final concentration 0.05-2.5 mM) was solubilised in 10 % v/v dimethylsufoxide (DMSO), final volume 20 µl. This was added to 20 µl of BBMV (final concentration 2 mg/ml). The mixture was incubated for 1 hour at 37 ^{O}C and the appearance of the chromogenic product *p*-nitroaniline was measured at 410 nm.

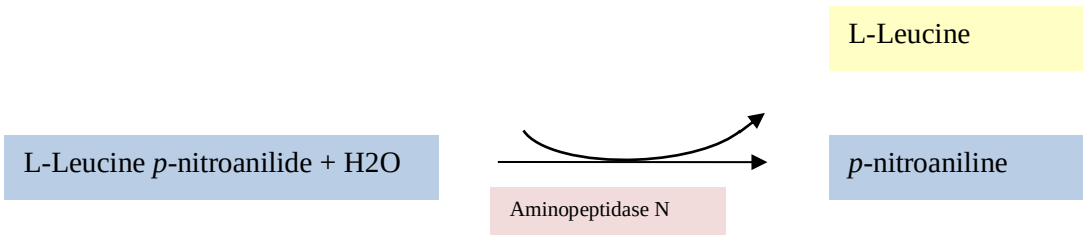

Figure 12. Enzymatic reaction for the hydrolysis of Leucine p-nitroanilide by APN

3.5.2 Neprilysin

Neprilysin was assayed according to a two-step method (Hersh and Morihara, 1986), figure 13. Neprilysin hydrolyses the bond between the proline and leucine residues of *N*-Suc-Ala-Ala-Pro-Leu-*p*NA (*i.e.* SAAPL-*p*NA). The second step of the assay involved BBMV APN, present in the admixture, hydrolysing the L-*p*NA bond, resulting in the release of leucine and the *p*-nitroaniline chromophore. 20 µl of SAAPL-*p*NA (final concentrations 0.07-20 mM), solubilised in 10 % v/v DMSO, was added to 20 µl of BBMV (final concentration 17 mg/ml). The mixture was incubated at 37 ^{O}C and NEP activity was stopped by the addition of phosphoramidon, final concentration 1.92 µM. The first step of the NEP assay involved incubating for 60 minutes and the second for 360 minutes due to the slowness of the reaction and gradual colour development. The appearance of the chromophore *p*-nitroaniline was measured at 410 nm.

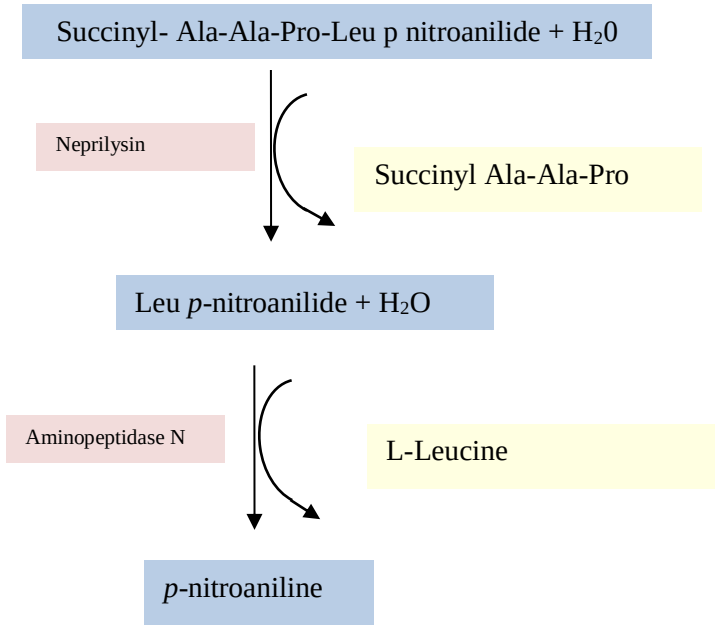

Figure 13. Two step enzymatic reaction for the hydrolysis of Suc-Ala-Ala-Pro-Leu-*p*-nitroanilide by NEP and APN.

3.5.3 Oligosaccharidases

The assays for sucrase, isomaltase, maltase, glucoamylase and lactase were based on the glucose oxidase/peroxidase reaction (Dahlqvist, 1964). For this work the GOPOD D-glucose determination kit (Megazyme International) was used to measure the appearance of D-glucose liberated during the hydrolysis of carbohydrates. Glucose oxidase converts liberated D-glucose into D-gluconate and peroxide; the peroxidase converts peroxide, *p*-hydroxybenzoic acid and 4-aminoantipyrine into the colorimetric product quinoneimine (figure 14).

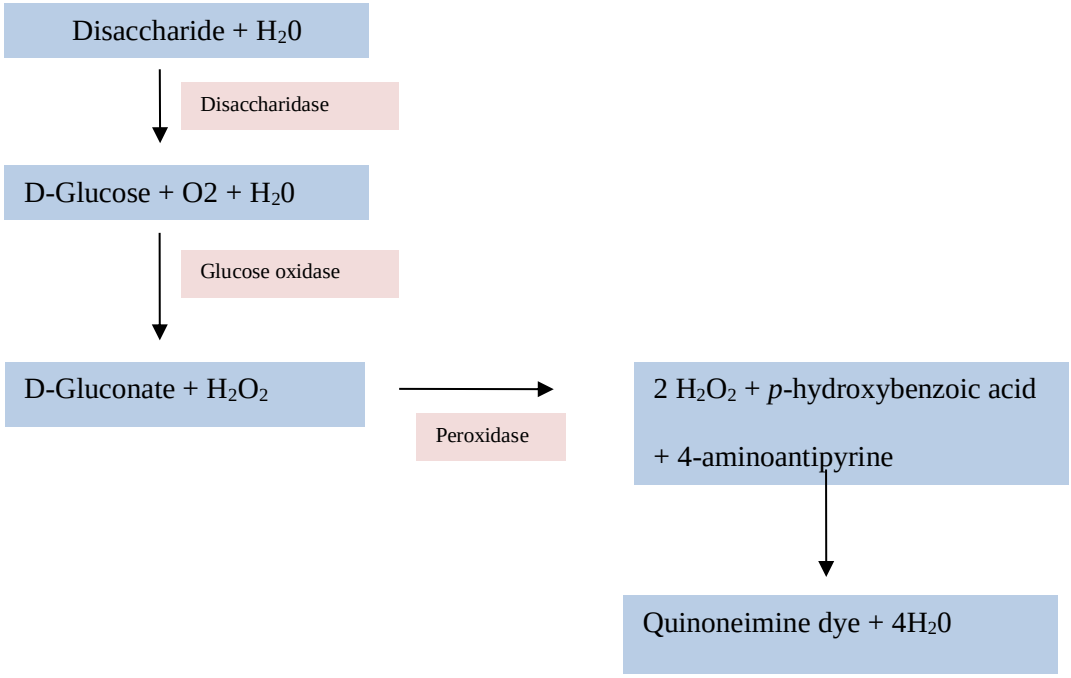

Figure 14. Enzymatic reactions involved in the oligosaccharide hydrolysis, glucose oxidase/peroxidase coupled reactions.

The substrate concentrations and incubation times for the oligosaccharidases were: sucrase (sucrose, 0.19 - 250 mM, 30 minutes), for isomaltase (isomaltotriose, 0.19 - 25 mM, 10 minutes), for maltase (maltose 0.09 - 10 mM, 10 minutes), for glucoamylase was pullulan (final concentration 0.19-25 mM, 960 minutes) and for lactase (lactose, 2.9 - 375 mM, 60 minutes). For each assay 20ul of enzyme and 20 µl of substrate were incubated for their respective times. 60 µl of GOPOD was then added and the plates were incubated

for 20 minutes at 50 ^{O}C, before the absorbance of the colorimetric product was determined at 510 ηm.

3.5.4 Alkaline phosphatase

Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as a substrate. Alkaline phosphatase hydrolyses the phosphate group liberating the colorimetric product *p*-Nitrophenol (figure 15). 20 µl of BBMV preparation and 20 µl of substrate (highest concentration 5.4 mM) were incubated together for 15 minutes, after which the assay was stopped with 40ul 0.1 M NaOH. The absorbance was then read at 405 nm.

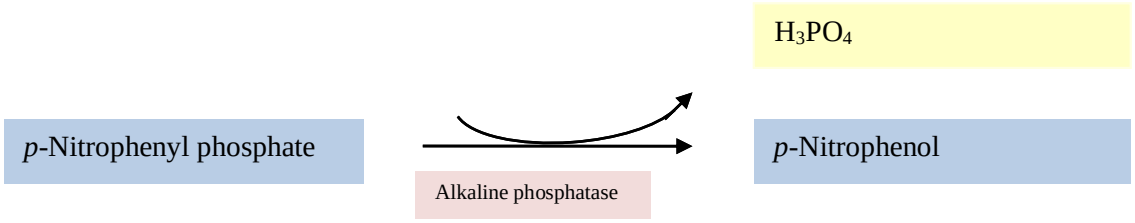

Figure 15. Enzymatic reaction for the hydrolysis of *p*-Nitrophenyl phosphate

3.6 LCMS method for identifying polyphenolic compounds

The samples were processed by Tony McGhie who developed and optimised the method. The samples were prepared, and the data analyses were performed by the author.

The LC-MS system was composed of a Dionex Ultimate® 3000 Rapid Separation LC system and a micrOTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray source operating in positive mode. The LC system contained a SRD-3400 solvent rack/degasser, HPR-3400RS binary pump, WPS-3000RS thermostated autosampler, and a TCC-3000RS thermostated column compartment. The analytical column was a Zorbax^{TM} SB-C18 2.1 x 100 mm, 1.8 µm (Agilent, Melbourne,

Australia) maintained at 50 °C and operated in gradient mode. Solvents were A = 0.5 % formic acid, and B = 100 % acetronitrile at a flow of 400 µL/min. The gradient was: 70 % A, 30 % B, 0-0.5 min; linear gradient to 45 % A, 55 % B, 0.5-25 min; linear gradient to 2 %A, 98 % B, 25-45 min; composition held at 2 % A, 98 % B, 45-50 min; linear gradient to 70 % A, 30 % B, 50-50.2 min; to return to the initial conditions before another sample injection at 54 min. The injection volume for samples and standards was 2 µl. The micrOTOF QII source parameters were: temperature 200 ºC; drying N_2 flow 8 L/min; nebulizer N_2 4.0 bar, endplate offset -500V, capillary voltage -4,000 V; mass range 100-1,500 Da, acquired at 2 scans/s. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis. Quantum analysis was undertaken by the author using QuantAnalysis software, (Bruker Daltonics, Bremen Germany). The known molecular weight and retention times of the polyphenolic compounds of interest were used to identify polyphenolic compounds, and internal standards were used for some compounds to quantify their concentration,

4 Chapter 4. Methods. A realistic *in vitro* milieu for measuring Brush border enzyme activity

Previous chapters have shown that BB digestion has an important role to play in the digestion, and subsequent absorption of nutrients, and demonstrated that current *in vitro* methodologies do not incorporate a replete array of BB digestive enzymes into their designs. In the preceding chapter we discussed and formulated procedures for the isolation of viable BBMV preparations and assay of BB hydrolytic enzyme. In this chapter we build on earlier chapters by developing rationales for the adjunct incorporation of mammalian BBMV fractions onto current *in vitro* digestion methods to realistically extend their hydrolytic capability.

4.1 Aims: the development of the BB *in vitro* digestion model

- The first aim was to use data from the literature to decide on the concentration and volumes of physiological secretions seen *in vivo*.
    - Use these data to assay enzymes under conditions representative of the small intestine.
    - Use these data to estimate the ratio of all component volumes in order to design the BB *in vitro* digestion method.
- The second aim was to ensure the pH optimum for the BB *in vitro* digestion was physiologically relevant.
- The third aim was to ensure the timing of the BB in vitro digestion was physiologically relevant.

4.2 Components required for a BBMV *in vitro* model

The *in vitro* BB digestion model is designed to work as an adjunct to buccal, gastric and pancreatic digestion. It is assumed that the digestate entering the small intestine includes food, as well as buccal and gastric secretions. Buccal enzymes are unlikely to retain function following acidification and peptidolysis in the stomach (Pedersen et al., 2002). Upon entering the small intestine the pH of gastric contents are rapidly neutralised, which can lead to eliminate, or dramatically reduce, gastric enzyme activities, *e.g.* pepsin activity is nominal at pH 7 (Piper and Fenton, 1965). Gastric lipase may continue to hydrolyse lipids under the neutral pH of the small intestine, but interfacial binding would be inhibited (Chahinian et al., 2006), so would reduce lipolysis. Since buccal and gastric enzyme activities are effectively stopped upon reaching the small intestine this work will focus on the concentration of biliopancreatic, and BB digestive secretions. In addition to the concentration of digestive secretions, one must examine the composition and ratio of digestive components entering the small intestine, and influencing volume changes within the small intestine.

There are three digestive components contributing to volume changes within the small intestine:

1. **Endogenous enzymes:** These include buccal, gastric, pancreatic and BBMV.
2. **Endogenous fluids:** These include salivary, mucoid and alkaline fluids secreted along the gastrointestinal tract.
3. **Exogenous sources:** Volumes associated with food boli and ingested liquids

The following rationales use published data to establish the ratios of endogenous and exogenous secretory components, and biliopancreatic digestion was modelled using

physiological concentrations and ratios of bile and pancreatic enzymes. So care should be taken when using methods with non – physiological concentrations. Section 4.2 presents the rationale and method used during preliminary work while section 4.3 presents is the rationale and method used for BB *in vitro* digestion

4.3 Components based on *in vivo* biliopancreatic and BB secretions

In this experiment the volumetric contribution of the meal, buccal, gastric and Brunner's gland secretions were not accounted for. During digestion there is an inconsistent flux of fluid from the stomach into the small intestine, from the small intestine into systemic circulation and from the small into the large intestine. As a result there will be significant changes in concentrations of enzymes and bile throughout the small intestine as the water content of digesta decreases from the proximal to distal small intestine, *e.g.* water is absorbed from the small intestine of the rat at a rate of 182 µl/cm/hr (Fisher, 1955). Hence, the principal focus was ensuring that the ratios of pancreatic enzymes, and bile, to the *succus entericus* were physiological at a given time point. This rationale was used to assess how bile and pancreatin affected BB enzyme activities (temporal assay) (**Chapter 6**) and these ratios were used to solubilise BBMV-bound enzymes in order to assess their kinetics at different pH's (**Chapter 7**).

4.3.1 Estimating the volume and concentration of pancreatin from *in vivo* data

The volume of pancreatic secretions was calculated for the first hour of the post-prandial period. The volume of pancreatic secretions was taken from the volume of secretin induced fluid collected from the cannulated pancreatic ducts of human participants, *i.e.* 248 µl/kg/5 min (Domschke et al., 1977). This volume was extrapolated for a 70 kg person, equating to 208.32 ml/hr (Domschke et al., 1977). The concentration of pancreatic

enzymes was based on published data that quantified the amylolytic activity of pancreatic juice; again collected via a cannula from the pancreatic ducts of humans during the post-prandial period (Ekmekcioglu, 2002), *i.e.* 55 U amylase activity are secreted per kg every 15 min following consumption of a 160 kcal meal (Ekmekcioglu, 2002). Extrapolating this equates to 15400 U or 63910 USP (1 U = 4.15 USP) of amylase activity per hour post-prandially for a 70 kg person. Pancreatin contains no less than 25 USP per mg and our pancreatin containing 8 x USP (US Pharmacopeia) pancreatin (p7545) contains 200 USP per mg. Hence, 63910 USP was equivalent to 355.19 mg of pancreatin/hr. Divided by the hourly secretion the concentration is calculated to be 1.53 mg of pancreatin per ml of buffer 2. This was in keeping with works, which show that pigs secrete 210 ml of pancreatic secretions per hour in the post-prandial period (Thaela et al., 1995), and that humans secrete 1-1.5 L of pancreatic fluid per day (DeSesso and Jacobson, 2001) For the purposes of this work porcine pancreatin was used to simulate pancreatic enzymes.

Calculation for pancreatin concentration

- Need 15400 U of amylase from porcine pancreatin
- Amylase: 4.15 USP = 1 U; Lipase 1 USP = 1 U (Littlewood et al., 2006)
- Pancreatin (Sigma Aldrich, P7545) 1mg of 8x USP = 25 USP units of amylase x 8 = 200 USP units per mg.
- Need 63910 USP
- Pancreatin: 200 USP units of amylolytic activity per mg of pancreatin: 63910/200
- Need 319.55mg of pancreatin per hour of secretion
- 319.55mg/208.32ml pancreatic secretions = 1.53398 mg/ml.

Table 7. The ratio and concentration of small intestinal digestive secretions used to emulate small intestinal conditions

	Micellar bile preparation	**Pancreatic enzyme preparation**	**BBMV and aqueous preparations**
Initial concentration (mg/ml)	20 (Begley et al., 2005)	1.53 (Ekmekcioglu, 2002)	5[#]
Final concentration (mg/ml)	0.72	0.39	4
Volume (ml)	29 (Kararli, 1995)	208 (Domschke et al., 1977)	572 (Mosenthal, 1911, de Beer et al., 1935)
Ratio	0.04	0.26	0.71

the concentration of the BBMV and aqueous preparations were equivalent to a starting concentration of 5 mg of mucosal scrapings per millilitre buffer.

4.3.2 Estimating the volume and concentration of bile from *in vivo* data

Like pancreatic exocrine secretions biliary fluid is continuously secreted and admixed with chyme exiting the stomach. Hence, estimates were based on the first hour of secretion. This method was modified to use crude bile salts, which are a composite of bile

acids in their natural concentrations, and so are most likely to reflect the range of bile salts present *in vivo*.

To make the biliary solution dried porcine bile salts were reconstituted with decanoic acid and salts into a micellar solution (Lentle et al., 2012). Porcine bile extract was added at concentration of 20 mg/ml (Begley et al., 2005) in a volume derived from published work of 0.415 (range 0.10-0.93) ml/kg/day (Kararli, 1995), and extrapolated to a hourly rate of 29 ml/hr.

Calculations for the preparation of micellar bile solution

- Human bile secretion 29 ml/hr (Kararli, 1995)
- 580 mg porcine bile extract per 29 ml or 20 mg/ml (Begley et al., 2005)
- 6 mM KCl (74.55 g/mol) = 0.0089 g/20ml (Lentle et al., 2012)
- 120 mM NaCl (58.44g/mol) =0.14025 g/20 ml(Lentle et al., 2012)
- 20 mg/ml Bile acids = 0.4 g/20ml
- Decanoic acid (172.26 g/mol) = 0.0034452 g/20 ml (Lentle et al., 2012)
- Add bile acids, salts, and decanoic acid to deoinised water and stir for 1 hour at 40 ºC

4.3.3 Estimating the volume and concentration of BBMV secretions from *in vivo* data

The volume and concentration of BBMV cannot be realistically determined *in vivo* due to variation along the length of the small intestine, and difficulty in determining the dilution of BBMV. Hence, BBMV may be more dilute in the intestinal lumen (McConnell et al., 2009) or more concentrated in the peri-apical space (luminal area surrounding the tips of microvilli), and in mucus secretions overlying the small intestinal surface. Moreover, all sites are difficult to harvest *in vivo* during the post-prandial phase. The total

volume of small intestinal BBMV secretions was estimated from published estimates of canine *succus entericus* secretions collected from Thiry Vela loops. Thiry Vela loops are small intestinal segments that are surgically isolated, cannulated and externalised, which leave an innervated section of the small intestine, which is not subject to admixture with gastric and pancreatic contents, from which *succus entericus* can be collected. The continuity of the small intestine was maintained via anastomosis. The isolated intestinal segments maintained blood supply and are innervated, so are responsive to normal neural and enterokine stimulation (de Beer et al., 1935, Mosenthal, 1911). These works are in general agreement that ~35ml of BB *succus entericus* is secreted per hour of the post-prandial phase from Thiry Vella loops of 25-50 cm length (de Beer et al., 1935, Mosenthal, 1911) in canines with a mean weight of 12.84 kg (*i.e.* 2.73 ml/kg/hr) (Mosenthal, 1911). The canine small intestine is approximately 225 - 290 cm long (Kararli, 1995), and the volumes secreted from the Thiry Vella loop differ greatly between intestinal segments, individuals and sampling points (Mosenthal, 1911, de Beer et al., 1935) so extrapolating, section for section, to match those secreted normally is difficult. Hence, it was assumed that each section of the small intestine secreted 35 ml of *succus entericus* totalling 105 ml/hour (8.18 ml/kg/cm) (Mosenthal, 1911). Extrapolated for a 70kg human this would be 572 ml/hour. With the estimated average daily secretions of *succus entericus* in humans of 1-2 litres per day (DeSesso and Jacobson, 2001) this estimate seems reasonable if associated with one meal.

Since there is no published data on the activity of BBMV enzymes *in vivo*, in the absence of biliopancreatic secretions, the concentration of BBMV used for the assay of BB enzymes was chosen to produce enzyme activities that could be measured by colorimetric assay.

4.3.4 Estimating the volume and concentration of the aqueous fraction

Described here is the rationale for the volume and concentration of the BB aqueous enzyme preparation. The aqueous fraction was recovered during the preparation and isolation of the BBMV fraction. It is likely to contain immature BB enzymes from the enterocyte, soluble BB enzymes and enzymes solubilised during the preparation of the BBMV fraction. There are no reliable estimates for the concentration of the aqueous fraction in the *succus entericus*. Since, enterocytes migrate to the tip of microvilli and are shed into the peri-apical space it is likely their contents are dispersed. Without appropriate data this fraction was diluted in the manner of the BBMV fraction.

4.3.5 Rationale for the admixture of component fractions

Bile and/or pancreatin were added to the BBMV and BB aqueous in the ratios outlined in table 7. These enzyme admixtures were either used for assay immediately or were held at 37 ^{O}C for 1 hour or 2 hours prior to adding the substrate. This enabled the time at which enzyme activities were modified by bile and pancreatin to be determined. Controls included enzyme and no substrate, substrate and no enzyme, heat treated enzyme and substrate, pancreatin ± bile, and pancreatic α-amylase as a positive control. In the latter the concentration of α-amylase was that which has been reported in pancreatin, *i.e.* 15400U (Ekmekcioglu, 2002) or 7.39 mg/ml.

4.4 Adjustments based on fluid volumes

In addition to physiological concentrations and volumes of biliopancreatic and BB secretions, adjustments were made in order to emulate endogenous and exogenous digestive components that contribute to volume changes within the small intestine. Again, the flux of fluid from the stomach to the small intestine, and from the small intestine into

systemic circulation was not modelled in this method. The rationale for admixture of gastrointestinal secretions, and the approximate volumes and concentrations of those secretions were taken from published data (table 8). Salivary secretions were not included as they were incumbent in the measured gastric volumes, as all saliva produced is generally swallowed (Chen, 2009, Pedersen et al., 2002).

This rationale was used to determine the kinetic activities of enzymes in the BBMV preparation under the conditions to be used in the *in vitro* digestion method (**Chapter 5**), was used to assess the impact of bile and pancreatin on BB enzymes in the exposure assay (**Chapter 6**), and for the *in vitro* digestion of polyphenolic compounds (**Chapter 8**).

Table 8. Estimated volumes of various gastrointestinal secretions associated with a fasted state and following a 1326 kj meal (14 % protein, 55 % CHO, 31 % fat)

Secretion	Volume (ml)	Reference
Inter-digestive gastric volume	237	(De Schepper et al., 2004)
Post-prandial gastric secretion	164	(De Schepper et al., 2004)
Meal size	311	(De Schepper et al., 2004)
Brunner's secretion	30	(DeSesso and Jacobson, 2001)
Pancreatic secretions	208	(Domschke et al., 1977)
Bile	112	(DeSesso and Jacobson, 2001)
Intestinal wall secretion (BBMV) (ml)	223	(DeSesso and Jacobson, 2001)
Total volume	1285	

4.4.1 Estimating the volume and concentration of exocrine pancreatic secretions from i*n vivo* data

The concentration and volume of pancreatic secretions were the same as section 4.21, *i.e.* 208 ml of exocrine pancreatic secretions (pancreatin) at a concentration of 1.53 mg/ml.

4.4.2 Estimating the volume and concentration of biliary secretions from *in vivo* data

The concentration of bile salts remained the same as in section 4.2.2, but as all endogenous and exogenous secretions were accounted for the concentration of bile was adjusted to suit the new method. The volumes attributed the meal, buccal, and gastric secretions were taken from a report that used single photon emission computed tomography (SPECT) to measure gastric volume changes during the pre-prandial (inter-digestive period when of relative inactivity) and post-prandial (following a meal) phases (De Schepper et al., 2004). Since the energy of the meal was given the volume of biliary secretions was extrapolated in relation to a model meal. The method for calculating the volume of biliary secretions involved two steps; the first used the energy composition of the meal to calculate its contribution to daily energy intake. Hence, the 1326 kJ meal (De Schepper et al., 2004) contributed 14.88 % to the average daily energy intake of an adult New Zealander of 8914 kj (University of Otago and Ministry of Health, 2011). The second calculation assumed that biliary secretions (750 ml/day) (DeSesso and Jacobson, 2001) were related to dietary energy so that the proportion of secretions could be calculated. Hence, the volume of biliary fluid secreted per meal was estimated to be 112 ml, *i.e.* 14.88 % of 750 ml (range 500-1,000/day) (DeSesso and Jacobson, 2001). The micellar preparation was then added in a concentration that was commensurate with known ratios of digestive secretions.

4.4.3 Estimating the volume of buccal and gastric preparations

The volume of secretions in the stomach were taken from a report using SPECT to measure gastric volume changes during the pre-prandial (237 ml) and post-prandial phases (475 ml) (De Schepper et al., 2004). Buccal and gastric secretions were incumbent in the pre-prandial, and post prandial volumes. Post-prandial gastric secretions were 164

ml while the volume of the meal was 311 ml (14 % protein, 55 % CHO, 31 % fat**)** (De Schepper et al., 2004). The meal consisted of solid and liquid components, *i.e.* one egg, a slice of brown bread, a pat of butter, 120 ml of orange juice and 25 ml of Ensure (De Schepper et al., 2004). It must be noted that the solid portion of the meal would not dilute the liquid phase, but as most meals are a mixed phase of solid and liquid these volumes were used as they were more physiologically relevant. Interestingly, the liquid phase of the meal induced a greater change in gastric volume than the mixed meal, *i.e*. 323 *vs.* 164 ml (De Schepper et al., 2004).

4.4.4 Estimating the volume of Brunner's gland preparations

No data regarding the volume of the post-prandial secretion of Brunner's glands was found although the daily secretory volume has been estimated to be 200 ml per day (DeSesso and Jacobson, 2001). Hence, the volume of Brunner's gland secretions had to be extrapolated in relation to the model meal. The method for calculating the volume of Brunner's gland secretions involved two steps; the first used the energy composition of the meal to calculate its contribution to daily energy intake. Hence, the 1326 kj meal (De Schepper et al., 2004) contributed 14.88 % to the average daily energy intake of an adult New Zealander of 8914 kj (University of Otago and Ministry of Health, 2011). The second calculation assumed that Brunner's gland secretions (200 ml/day) (DeSesso and Jacobson, 2001) were related to dietary energy so that the proportion of secretions could be calculated. Hence, this volume was calculated to be 30 mls, *i.e.* 14.88% of 200 ml, assuming that a) the volume accounts for the pre-and post-prandial Brunner's secretions associated with this meal and b) the volume is dependent on the energy content of a meal. Factors such as nutrient content, pH and composition of the meal will alter these and other secretions *in vivo*.

4.4.5 Estimating the volume and concentration of BBMV '*succus entericus*'secretions

The daily volume of BBMV secretions was again estimated from the volume of *succus entericus* fluid secreted per day, *i.e.* 1.5 L (range 1-2 L/day) (DeSesso and Jacobson, 2001) using the same method as that developed for the calculation of the volume of Brunner's gland secretions. Hence, the volume of BBMV was 223 ml, *i.e.* 14.88 % of 1.5 L. This ratio of BBMV used in experiment II was lower than that used in experiment I, *i.e.* 17 % *vs.* 35 % respectively. However, in order to ensure adequate activity the final concentration of the BBMV fraction remained the same, *i.e.* 2 mg/ml (wet weight) for all enzymes except glucoamylase and NEP (6 mg/ml).

4.5 *In vitro* simulation of BB enzyme fractions found *in vivo*

4.5.1 Rationale for the solubilisation of BBMV-bound enzymes

Brush border enzymes may remain attached to the surface of BBMV or may be solubilised from the membrane by biliopancreatic secretions *in vivo*, which results in either amphipathic (bile solubilised) or a soluble (proteolytically solubilised) enzyme. Solubilisation of BB enzymes was important to investigate in order to determine whether solubilisation has a deleterious effect on BB enzyme activities. Hence, in chapter 7 BBMV-bound enzyme activities were compared with pancreatin solubilised (soluble fraction), bile solubilised enzymes (amphipathic fraction), and cytosolic (aqueous fraction) enzyme activities. In order to solubilise BBMV anchored enzymes in a bio-relevant way it was necessary to use physiological concentrations and volumes of bile, pancreatic and BBMV secretions present in the small intestine (section 4.2).

4.5.2 Preparation of the BBMV enzyme fraction

Brush border membrane vesicles are actively shed into *succus entericus* (McConnell and Tyska, 2007) in an amount that has not been yet been quantified. Difficulties in quantification arise as BBMV are both diluted in the intestinal lumen (McConnell et al., 2009), and are likely to be localised to the peri-apical space and mucus overlying the epithelia. The rationale for the volume and concentration of BBMV are described in section 4.2.3. The initial concentration was 5.6 mg/ml (effective concentration of mucosal scrapings used to prepare the BBMV fraction) resulting in an enzyme admixture containing 4 mg/ml and a final concentration of 2 mg/ml.

4.5.3 Preparation of the aqueous enzyme fraction

Supernatant II was retained during BBMV purification as the aqueous fraction. It is comprised of water miscible enzymes originating in the cytosol of the enterocyte that are likely to be a mixture of intermediate forms of BB enzymes (Cezard et al., 1979, Maze and Gray, 1980), solublised BB enzymes entrapped in the mucus overlying the epithelia, and BB enzymes that have been mechanically solubilised during the process of purification (Maestracci, 1976). The aqueous fraction was diluted to the same concentration as the BBMV fraction.

4.5.4 Preparation of the soluble “pancreatin solubilised” BB enzyme fraction

Pancreatic peptidases were used to hydrolyse the stalked region of BB enzymes attached to BBMV. This liberated soluble BB enzymes with no transmembrane anchoring segments (Semenza, 1986).

Preparation of the soluble fraction

The volume and concentration of pancreatic secretions are detailed in section 4.2.1. The soluble fraction was prepared in batches of 10 mls; comprising of 7.1 ml of 5.6 mg/ml BBMV, 2.6 ml of 1.53 mg/ml pancreatin and 0.4 ml of buffer 2. The fractions were mixed and held for 1 hour at 37 ^{O}C before centrifugation at 27,000 x *g* for 30 minutes at 4ºC, the supernatant was retained as the soluble fraction.

4.5.5 Preparation of the amphipathic "bile solubilised" BB fraction

The amphipathic fraction comprised BB enzymes solubilised by bile. This fraction differs from the soluble fraction in that the enzymes maintain their transmembrane anchor, and so has amphipathic properties.

Preparation of the amphipathic fraction

Crude bile salts were reconstituted into a micellar preparation (Lentle et al., 2012), see section 4.2.2. The amphipathic fraction was prepared in volumes of 10 mls; 400 µl of 20 mg/ml micellar preparation was added to 7.1 ml of BBMV and 2.1 ml of buffer 2 and held for 1 hour at 37 ^{O}C. The fraction was then centrifuged at 27,000 x g for 30 min at 4ºC, and the supernatant retained as the amphipathic fraction.

4.6 Conclusion

The preceding rationales provide physiological justification for the ratios and concentrations of endogenous and exogenous fluid volumes used in subsequent chapters. Experimental work was undertaken using these principals to determine whether the BBMV fraction was appropriate for use *ex vivo* in an environment representative of the small intestine.

5 Chapter 5. The kinetics of small intestinal brush border enzymes

This chapter describes an investigation of the kinetics of seven enzymes that are associated with BBMV obtained from small intestinal mucosa: maltase, glucoamylase, sucrase, isomaltase, lactase, aminopeptidase N (APN) and neprilysin (NEP). Quantification of enzyme kinetics were necessary to provide a means by which to standardise BBMV fractions from different *ex vivo* preparations. Hence, the inter-assay variability of the various kinetic values of these enzymes was assessed and contrasted with published values for BBMV-bound enzymes of the rat small intestine. Further, knowledge of the K_m and V_{max} of a particular enzyme or consortia of enzymes was useful for assessing the volume of enzyme preparation that would be required to efficiently degrade macronutrients in food. Hence, where an enzyme has high affinity (low K_m) for a substrate, maximal velocity (V_{max}) will be achieved with a low concentration of enzyme. Then if the concentration of a particular substrate in the small intestine (*e.g.* sucrose), and its likely dilution in digestate are determined, we can calculate whether its concentration in digesta would be enough to achieve V_{max} with this enzyme concentration. If this process is continued for a number of substrates we can determine whether the ratio of K_m and V_{max} values for these enzymes, within the purified BBMV, reflects the concentrations of substrates within the normal diet. Hence, the volume of BBMV enzymes that is sufficient to achieve V_{max} for the individual substrates could be calculated.

Therefore, the aims of this chapter are threefold. Firstly, to define the kinetics of various BBMV enzymes, secondly to determine the likely concentration of these substrates found in the normal digesta of the rat, and finally to determine whether the BBMV are efficiently hydrolysing these substrates.

5.1 Materials and methods

5.1.1 Reagents

See section 3.1.

5.1.2 Preparation of the BBMV fraction

Mucosal scrapings were obtained from the small intestine of eight 10 week old male Sprague Dawley rats via dissection (AEC 1364). The husbandry and procedures are described in detail in section 3.2.

The small intestinal mucosae of eight rats were pooled for preparation of the BBMV fraction using a modified version of the calcium precipitation and differential centrifugation method (Boutrou et al., 2008, Kessler et al., 1978, Sakuma et al., 2009). The full description is given in section 3.3.2, and is depicted in figure 4.

Where different BBMV preparations were used for the assay of BB enzymes they are assigned an identifying label, such as APN1, APN2 and APN3 for the three repeats of APN that used BBMV originating from 3 groups of rats, with 4 replicates at each substrate concentration.

5.1.2.1 Assay details

The assay methods were chosen for their ability to be scaled to a 384 well plate, with methods detailed in section 3.5. Absorbance's were determined with a SpectroMax Plus spectrophotometer (Biostrategy, Auckland, NZ) with Softmax Pro analysis software 3.0 and Griener (GR7811010) 384 well microplates. The incubation time, and the concentration of substrates and the BBMV fraction used in these assays are shown in table 9.

Table 9. Substrate, enzyme concentration, and incubation times used for the kinetic investigation of BBMV-bound enzymes

Enzyme	Maltase	Sucrase	Lactase	Isomaltase	Glucoamylase	APN	NEP
Substrate	Maltose	Sucrose	Lactose	Isomaltotriose	Pullulan	Leu-*p*NA	SAAPL-pNA
Highest concentration (mM)	10	250	375	25	7.5*	2.5	20
BB enzyme concentration (mg/ml)#	2	2	2	2	17	2	17
Incubation time (min)	10	30	60	10	960	60	60/360

Key: * mg/ml; #: The final concentration of BBMV fraction. The concentration reflects the original concentration of mucosal scrapings in the homogenate from which BBMV fraction was isolated

5.1.3 Calculation of nutrient concentrations within the small intestine

The metabolic weight (MW) of these rats was calculated from their weight using the calculation: **Weight (g) ^ $^{0.75}$**. The volume of fluid entering the rat small intestine was extrapolated from human data (Sherwood, 2011) as quantification or estimates of all endogenous rat secretions were not found. These volumes were used to calculate the volume of fluid that enters the rat small intestine daily (calculation 1).

Calculation 1:

Rat metabolic weight (weight (g) ^$^{0.75}$) * volume of intestinal fluid (human) per gram metabolic weight (ml/g MW per day)

Estimates of rat AIN93G (standardised diet) intake (Kenar et al., 2007, Chang et al., 2006, Gallaher et al., 2000) and composition (Reeves et al., 1993) were used to calculate the likely concentration of nutrients in the small intestine. The estimated concentrations of

these nutrients were graphed alongside the enzyme activities of relevant enzymes to assess the functioning of these enzymes.

5.1.4 Characterisation of enzyme kinetics

The activities of the enzymes were assayed over a series of substrate concentrations and the activities (pmol/min) at each substrate concentration were plotted and used to determine the K_m and V_{max} for each enzyme. Non-linear curve fitting was conducted using the Origin graphing programme (OriginLab, Northampton, MA) to fit Hill: y = V_{max} *x^n / (k^n+x^n) (the fitted line passes through zero) or Hill 1 curves: y = START + (END - START) * x^n / (k^n + x^n) (where the line does not pass through zero).

5.1.5 Statistical analysis

Data were obtained from kinetic experiments in which enzyme activities were assayed over a series of substrate concentrations (n=4). The data were used to determine the K_m and V_{max} for each enzyme using the Origin graphing program. The values obtained for K_m and V_{max} were compared by Students T-tests. ANOVA could not be used as the K_m and V_{max} values given by Origin are estimates rather than true means. Origin estimates the kinetic parameters using an iterative process, *i.e.* the best fit is obtained by minimising the Chi-squared values of all the parameters.

5.2 Results

5.2.1 General

The plots for the rate (pmol/min) of BB enzymes were generally curvilinear in form, and had R-squared values, for fit, of between 0.913-0.995. The initial parts of graphs were generally linear up to the point of inflexion. In some cases there was a tendency for the

activities to decline with higher substrate concentrations, notably in Gluc1, Gluc2 (figure 19) and malt 1 (figure 22).

5.2.2 Nutrient concentrations within the rat small intestine

The mean weight of the rats was 417 ± 17.8 g, the MW was calculated to be 92.28 g, and the volume of fluid entering the small intestine per day was calculated to be 192 ml. Since rats consume about 8.5 g of AIN93G per 100 gram bodyweight/day they were estimated to consume an average of 35.45 g per day. Allowing the concentrations of nutrients in the small intestine to be estimated, table 10.

Table 10. Estimated concentration of nutrients present in the small intestine of the rat

Enzyme	Substrate	Concentration (mM)
Sucrase	Sucrose	55
Maltase	Maltose	245
Isomaltase	Isomaltotriose	4.8
APN	Leucine/isoleucine	1.5
NEP	Proline-Leucine	11

5.2.3 Differences in the kinetic activities of enzymes between BBMV preparations

5.2.3.1 Neprilysin

There were significant differences between the K_m (d.f. 1,90, t=8.32, p<0.0001) and V_{max} (d.f. 1,90, t=19.97, p<0.0001) of BBMV preparations for NEP, (figure 16/table 11). The slower conversion of S-AAPL-*p*NA to the chromogenic product in NEP2 resulted in the assay requiring a longer comparative incubation time than NEP1. This was reflected in the lower V_{max}, *i.e.* 45 pmol/min *vs.* 144 pmol/min. In summary, there was a significant difference between NEP1 and NEP2 in their affinity for S-AAPL-*p*NA, and the

differences in hydrolysis rate resulted in significantly different maximal velocities. However, both were within the same order of magnitude. With the estimated concentration of internal proline-leucine residues in the diet being 11 mM, it appears that NEP in both groups NEP would be functioning at $V_{max.}$

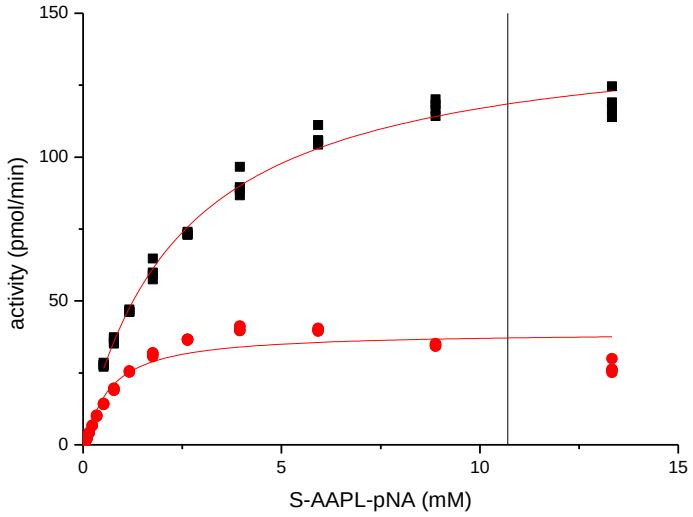

Figure 16. Graph showing the non-linear curve fits for Neprilysin.
Black: NEP 1, red NEP 2. The vertical line at 10.71 mM depicts the estimated concentration of …X-Pro-Leu-X… oligopeptides present in the small intestine.

Table 11. Comparison of the kinetic activity (K_m and V_{max}) of Neprilysin in BBMV fractions.

Identifier	K_m (mM)	V_{max} (pmol/min)	R^2 value	Model
NEP1	2.48(0.25)[a]	144(4.53)[a]	0.989	Hill 1
NEP2	0.52(0.10)[b]	45(2.71)[b]	0.911	Hill 1

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of V_{max} used the Hill 1 equation: y = START + (END - START) * x^n / (k^n + x^n); information following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by unpaired T tests (α=0.05).
#information in brackets is the standard error

5.2.3.2 Aminopeptidase N

There were significant differences in the kinetic activities of APN from different BBMV fractions, figure 17, and table 12. Firstly, the K_m values were all significantly different. The K_m for APN1 was twice that of APN2 and APN3, *i.e.* 0.67 and 0.23 and 0.17 mM respectively. Hence, APN1 was significantly different to APN2 (d.f. 1,94, t=2.41, p=0.0178) and APN3 (d.f. 1,86, t=2.56, p=0.0123). While APN2 was significantly different from APN3 (d.f. 1,102, t=2.35, p 0.0206). The maximal velocity of APN1 was also significantly greater than APN2 (d.f. 94, t=5.80, p=0.0001), and APN3 (d.f. 1,86, t=5.40, p=0.0001) respectively. The maximal velocity of APN2 was not significantly different from APN3. Since, AP1 showed less affinity for its substrate it would require higher concentrations of substrate to reach the same level of activity, but when substrate concentrations exceed 0.79 mM, activities increase 1.5 fold compared to APN2 or APN3 suggesting a greater turnover.

Again, as the likely concentration of Leu-x in the small intestinal lumen is thought to be 1.53 mM, all BBMV fractions measured would be functioning at $V_{max.}$

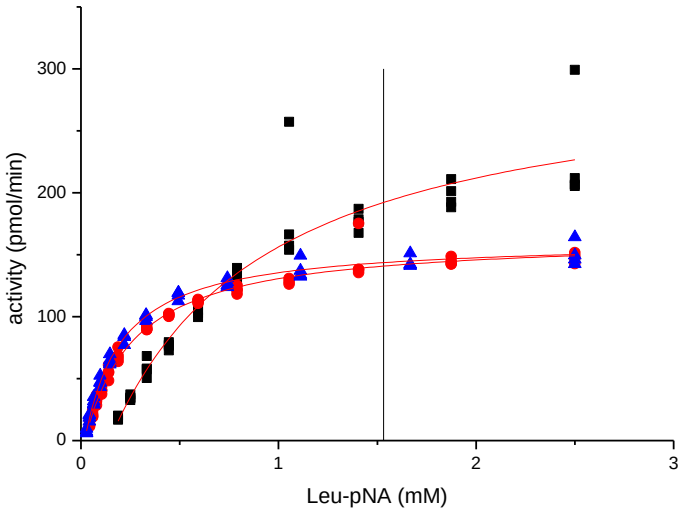

Figure 17. Graph showing the non-linear curve fits for Aminopeptidase N.
APN1: black, APN2: red, APN3: blue. The vertical line at 1.53 mM depicts the estimated concentration of P_1 Leu in oligopeptides present in small intestinal contents following pancreatic digestion

Table 12. Comparison of the kinetic activity (K_m and V_{max}) of APN in BBMV fractions.

Identifier	K_m (mM)	V_{max} (pmol/min)	R^2 value	Model
APN1	0.67 (0.214)[a]	370 (38.37)[a]	0.913	Hill 1
APN2	0.23 (0.022)[b]	179 (5.03)[b]	0.985	Hill 1
APN3	0.17 (0.012)[c]	180 (3.39)[b]	0.993	Hill 1

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of V_{max} used the Hill 1 equation: y = START + (END - START) * x^n / (k^n + x^n); information following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by unpaired T tests (α=0.05) #: information in brackets represents the standard error

5.2.3.3 Lactase

Again there were significant differences in the K_m values for lactase from different BBMV preparations, the K_m for Lac1 was significantly different to Lac2 (d.f. 1,110, t=2.70, p=0.008) and Lac3 (d.f. 1,97, t=3.39, p=0.001), (figure 18/table 13). Further, the K_m for Lac2 was significantly different to Lac3 (d.f. 1,105, t=5.64, p=0.0001). In contrast there were no significant differences in the maximal velocities of lactase. In summary, there were differences in the affinities of lactase for lactose with different BBMV fractions, but this did not result in significant differences in maximal velocities. There was no estimate of lactose concentration in the small intestine as AIN93G does not contain lactose.

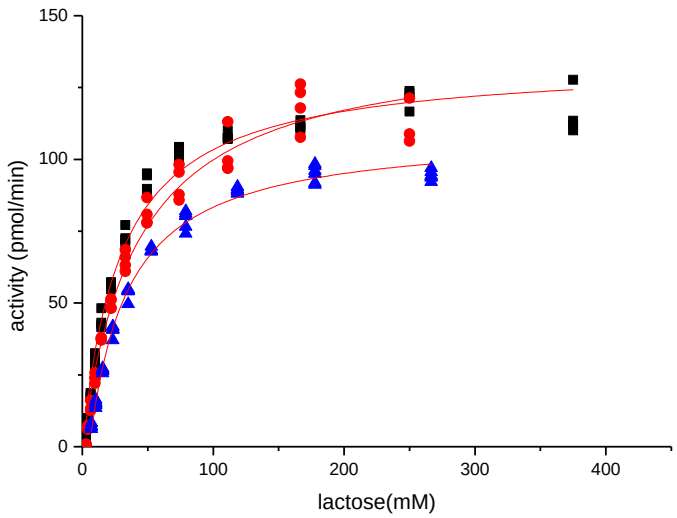

Figure 18. Graph showing the non-linear curve fits for lactase;
Lac1: black, Lac2: blue; Lac3: red.

Table 13. Comparison of the kinetic activity (K_m and V_{max}) of lactase in BBMV fractions.

Identifier	K_m (mM)	V_{max} (pmol/min)	R^2 value	Model
Lac1	30(1.79)[a]	134(2.39)[a]	0.979	Hill
Lac2	25(1.37)[b]	134(2.51)[a]	0.994	Hill1
Lac3	42(3.16)[c]	142(3.93)[a]	0.976	Hill

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of Vmax using the Hill 1 equation was y = START + (END - START) * x^n / (k^n + x^n); the Hill equation used was y= Vmax *x^n/(k^n+x^n). Information following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by T-tests (α=0.05). Information in brackets is the standard error.

5.2.3.4 Glucoamylase

Although there was a significant difference between the K_m values for glucoamylase from different BBMV preparations, Gluc1 being 2.5 fold lower than Gluc2 (d.f. 1,99, t=3.72 p=0.0003), there was no significant difference in the calculated V_{max} values, figure 19 table 14. In both cases maximal activities were decreased; when pullulan was at concentrations greater than 2 mg/ml there was a tapering of activities suggesting inhibition. In summary, there were significant differences in the affinities of

glucoamylase for pullulan in different BBMV preparations, but this did not result in differences in the maximum velocities.

The pullulan polymer is not present in the small intestine so there is no estimate of its concentration in the small intestine superimposed onto figure 19.

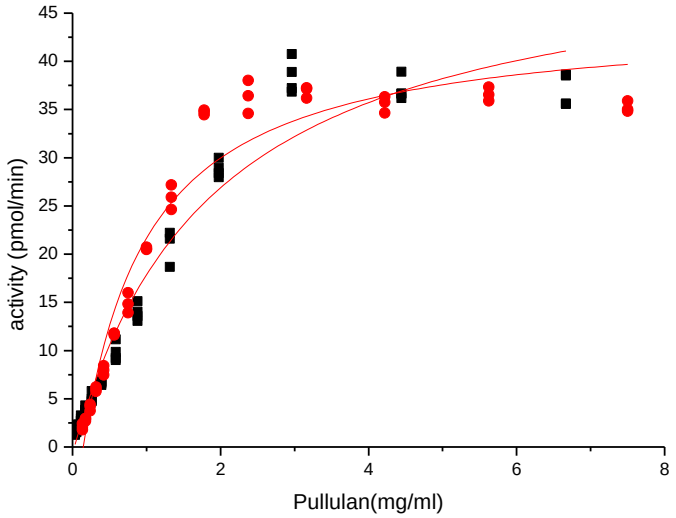

Figure 19. Graph showing the non-linear curve fits for Glucoamylase

Gluc1: Red, Gluc 2: black

Table 14. Comparison of the kinetic activity (K_m and V_{max}) of glucoamylase in BBMV fractions.

Identifier	K_m (mg/ml)	V_{max} (pmol/min)	R^2 value	Model
Gluc1	0.76(0.130)[a]	53(2.78)[a]	0.954	Hill 1
Gluc2	1.89(0.249)[b]	54(2.60)[a]	0.966	Hill 1

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of V_{max} used the Hill 1 equation: y = START + (END - START) * x^n / (k^n + x^n); information in brackets is the standard error of the mean; information following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by T-tests (α=0.05); information in brackets is the standard error of the mean.

5.2.3.5 Sucrase

There were no significant differences in the calculated K_m values of sucrase from different BBMV fractions, figure 20 table 15. Although there were no significant differences in the maximal velocities of Suc1 and Suc2, there were significant differences in the maximal velocities between Suc1 and Suc3 (d.f. 1,94, t=5.1, p=0.0001), and between Suc2 and Suc3 (d.f. 1,89, T=7.97, p=0.0001). Hence, although the affinity of sucrase for sucrose did not differ significantly between assays the enzyme did not reach the same V_{max} each time. In the small intestine the concentration of sucrose is thought to be 55 mM. Being that this is just above the K_m for sucrase the concentrations of sucrase found in the small intestine would not induce maximal velocity.

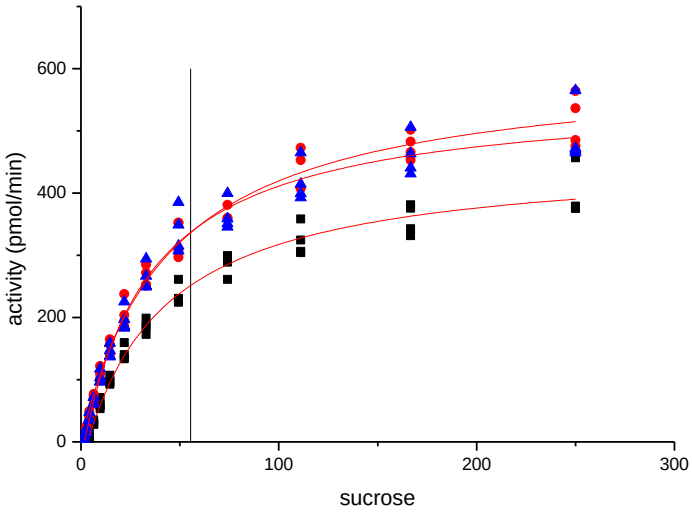

Figure 20. Graphs showing the non-linear curve fits for Sucrase.
Line:black: Suc3, Suc2: Red, Suc1: blue. The grey line at 55 mM represents the estimated concentration of sucrose in the small intestine.

Table 15. Comparison of the kinetic activity (K_m and V_{max}) of sucrase in BBMV fractions.

Identifier	K_m (mM)	V_{max} (pmol/min)	R^2 value	Model
Suc1	42(2.96)[a]	581(14.63)[a]	0.979	Hill
Suc2	45(2.52)[a]	606(12.51)[a]	0.986	Hill
Suc3	39(3.93)[a]	497(4.60)[b]	0.984	Hill 1

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of V_{max} using the Hill 1 equation was y = START + (END - START) * x^n / (k^n + x^n); the Hill equation used was y= V_{max} *x^n/(k^n+x^n). Information in brackets is the standard error of the mean. Information following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by T-tests (α=0.05).

5.2.3.1 Isomaltase

There were no significant differences between the K_m values for isomaltase from different BBMV fractions, *i.e.* 3.28 vs. 3.54 mM (figure 21, table 16). In contrast there was a significant difference in their maximal velocities, Iso1 had a 1.25 fold greater maximal velocity than Iso2 (d.f. 1,102, t=3.96 p=0.0001). Hence, like sucrase, there was no significant difference between the K_m values for isomaltase, but there were significantly different maximal velocities. Further, as the estimated concentration of glucose α-1,6 (*i.e.*isomaltotriose) is approximately 4.8 mM isomaltase may not be reaching V_{max} in the small intestine.

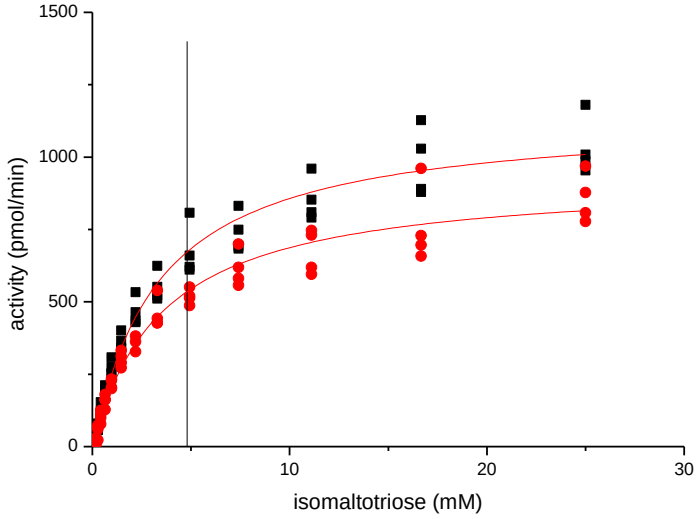

Figure 21. Graph showing the non-linear curve fits for Isomaltase.

Lines: black: Iso1, red: Iso2. The grey line at 4.8 mM represents the estimated concentration of isomaltotriose in the small intestine.

Table 16. Comparison of the kinetic activity (K_m and V_{max}) of isomaltase in BBMV fractions.

Identifier	K_m (mM)	V_{max} (pmol/min)	R^2 value	Model
Iso1	3.28(0.40)[a]	1161(41.83)[a]	0.969	Hill 1
Iso2	3.54(0.51)[a]	931(40.18)[b]	0.957	Hill 1

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of V_{max} used the Hill 1 equation: y = START + (END - START) * x^n / (k^n + x^n); information in brackets is the standard error. Iinformation following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by T-tests (α=0.05).

5.2.3.2 Maltase

There was a significant, 2.6 fold difference, between the K_m values for the hydrolysis of maltose (d.f. 1,94, t=2.58 p=0.0113) obtained from different BBMV fractions, figure 22 table 17. In addition, the maximal velocities for maltase were also significantly different (d.f. 1,94, t=2.32, p=0.0225); hence, a decrease in affinity (Malt2) was accompanied by an increase in maximal velocity suggesting a change to rate of substrate unbinding. Maltolysis is achieved by the action of four enzymes, which causes significant differences in maltase kinetics. The estimated concentration of α-1,4 bonds (maltose) from starch in the small intestine in response to AIN93G consumption was 245 mM; this could not be superimposed on figure 22 as maltose concentration did not exceed 10 mM. As such the concentration of maltose in the small intestine is 50 - 150 fold higher than the estimate K_m, which suggests that maltolysis is occurring at $V_{max.}$

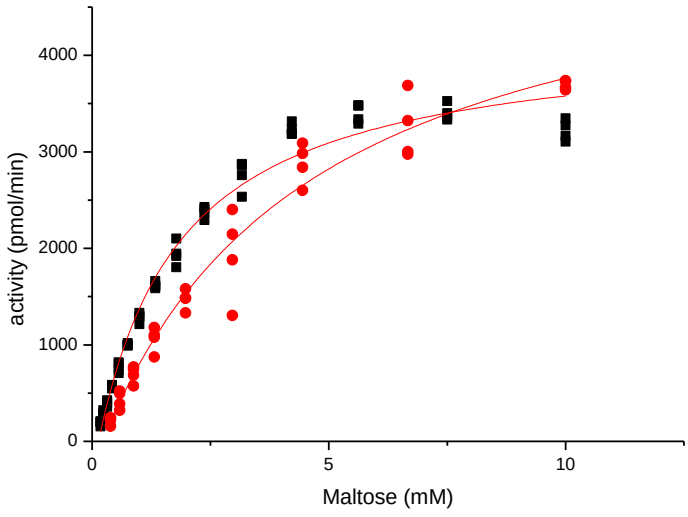

Figure 22. Graph showing the non-linear curve fits for Maltolytic enzymes.
Lines: Malt1: black, Malt2: red

Table 17. Comparison of the kinetic activity (K_m and V_{max}) of maltase in BBMV fractions.

Identifier	K_m (mM)	V_{max} (pmol/min)	R^2 value	Model
Malt1	1.65(0.15)[a]	4571(128)[a]	0.982	Hill 1
Malt2	4.34(0.80)[b]	5802(403)[b]	0.969	Hill 1

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of V_{max} used the Hill 1 equation: y = START + (END - START) * x^n / (k^n + x^n); information in brackets is the standard error. Information following the K_m and V_{max} values represents the standard error. K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by T-tests (α=0.05).

5.3 Discussion

5.3.1 Standardisation of activities

The enzyme activities described here were not standardised by BBMV weight or protein content, as is standard practice. Preparations of BBMV are not highly purified or homogenous and so BBMV weight or protein content do not give an accurate indication of the amount of enzyme present. As such, activities were not standardised against freeze dried BBMV weight due to the presence of salts, and mannitol in the buffer contributing to the dry weight of BBMV. Residue buffer also associates with the BBMV pellet, and the sides of the oakridge tubes, which in turn did not allow for the accurate measurement of the wet weight of BBMV pellets. For example 8 rats (n=8) with an average weight of 435 ± 12.58 g yield an average of 2.64 +/ 0.32 g of mucosal scrapings, which equates to an average BBMV pellet of 0.52 g (wet weight) per animal. Protein estimation of the mucosal homogenate or BBMV fraction was not used as it does not differentiate between individual proteins. Proteomic analysis shows that of the 646 proteins associated with BBMV with only 17 % of them being hydrolases (McConnell et al., 2011). Hence, for this work activities were given in pmol/min.

5.3.2 The concentration of nutrients within the small intestine

In order to calculate the concentration of nutrients present in the small intestine it was important to research the volume of endogenous and exogenous secretions contributing to small intestinal luminal volume. In rats the volume of pancreatic and biliary secretions are often measured when the animal is under anaesthetic (Louie et al., 1985, Niederau et al., 1989), but no information was found on the volumes of rat BB and Brunner's gland secretions. Instead the weight of metabolic tissue was determined, and the volume of fluid estimated from human data. The metabolic weight (MW) of a 70kg human was calculated to be 4303.52 g (using calculation in section 5.1.3). This estimate was used to calculate the volume of fluid entering the human small intestine per gram of MW.

The estimated volume of fluid entering, and being absorbed, by the human small intestine is thought to be approximately 9 L per day (Sherwood, 2011). Of this approximately 5.2 - 8.2 L are exogenous digestive secretions (DeSesso and Jacobson, 2001), the remaining is dietary water, *i.e.* approximately 3.3 L (Sawka et al., 2005). Since 9 L is an accepted estimate of fluid entering the small intestine per day this value was used to determine the ratio of digestive fluid per gram of MW per day, *i.e.* 2.09 ml/day/g MW. The volume of fluid entering the rat small intestine could then be calculated from the human estimate. Since the MW of the rat was calculated to be 92.28 g, the volume of fluid entering the small intestine was calculated to be 192.98 ml/day

In retrospect it would have been useful to have measured food intake so that the volume of nutrients in the small intestine could be precisely calculated. Instead the weight of AIN93G consumed by rats was estimated from published works. These show that rats, average weight 296.01 g, consume an average of 25.17 g of AIN93G per day (Kenar et al., 2007, Chang et al., 2006, Gallaher et al., 2000), *i.e.* 8.5g of AIN93G per 100 g bodyweight. Since the concentration of nutrients in AIN93G are known (Reeves et al., 1993) we can estimate the concentration of nutrients diluted in intestinal fluid. AIN93G

contains 100 milligrams of sucrose per gram so the average rat (417 g) would consume 3545.32 mg of sucrose per day, *i.e.* 55.34 mM. This is above the K_m values calculated for BBMV sucrase, *i.e.* 39 - 45 mM, but not near K_m associated with maximal velocity, approximately 250 mM. Although sucrose is a major component of AIN93G, sucrase would not be working at maximum velocity. Trace amounts of sucrose (and the digestive product fructose) are found in the rat large intestine (Dahlqvist and Thomson, 1963a) and in human ileostomy studies (Normén et al., 2001) suggesting that there may be incomplete hydrolysis of sucrose. The primary site for sucrase hydrolysis is the proximal small intestine, particularly the proximal jejunum (Cajori, 1933, Dahlqvist and Thomson, 1963b). As fluid is absorbed along the length of the small intestine (DeSesso and Jacobson, 2001) the concentration of sucrase in intestinal contents increases during small intestinal transit so that maximal activity could be acheived.

The primary nutrient in AIN93G is cornstarch. There are 529.5 milligrams of cornstarch per gram of AIN93G. Normal cornstarch contains approximately 90 %, carbohydrate (CHO) (Karkalas, 1985, Morrison et al., 1984, Chinnaswamy and Hanna, 1988) 0.3 %, 0.1 %, 9.5 %, 0.2 % of protein, lipid, water, ash (minerals) (Karkalas, 1985, Morrison et al., 1984, Chinnaswamy and Hanna, 1988) 1 % fibre (Foodworks) respectively. The amylose and amylopectin component of normal cornstarch is 25 % and 75 % respectively (Chinnaswamy and Hanna, 1988). Taking into account the proximate analysis, normal cornstarch would be comprised of 22.2 % amylose and 66.8 % amylopectin. Hence, the average rat would consume 5162 mg of amylose and 13610 mg of amylopectin per day. The α-1,6 branching of amylopectin occurs approximately every 25 glucose units (Thompson, 2000), *i.e.* 500.66 mg/day (4 % of amylopectin). Hence, 16188.06 mg of α-1,4 linked glucose residues would be consumed per day. If we assume these weights equate to specific nutrients we can calculate a molar concentration. Hence, the

concentration of maltose in luminal contents was approximately 245 mM and the concentration of isomaltotriose was 4.8 mM. The estimated small intestinal concentration of maltose is greater than the K_m, *i.e*. 3.28-3.54 mM. If maltase functions similarly *in vivo* then it would be working at concentrations that would ensure maximal activity, but at concentration of 245 mM maltose may cause substrate inhibition (Dahlqvist, 1960b), and the product glucose may also cause enzyme inhibition (Semenza, 1969), which may alter the activities of maltolytic enzymes. Further, unless glucose is rapidly absorbed, transglycosylation of the resulting monosaccharides can also occur (Zagalak and Curtius, 1975, Semenza, 1969). However, the hydrolysis of 245 mM of maltose would result in the production of glucose at levels similar to those seen during absorption, *i.e*. glucose concentrations of 200-300 mM (Pappenheimer, 1993). Glucose produced is absorbed via transcellular and paracellular pathways (Pappenheimer, 1993, Shirazi-Beechey et al., 2011) so would not be expected to cause product inhibition *in vivo*. It is important to note that removal of product during *in vitro* digestion may promote the continued hydrolysis of oligosaccharides.

It is difficult to calculate the likely concentration of *β*-casein oligopeptides in the small intestine as there are two consortia of enzymes (pancreatic and BB) that hydrolyse these peptides. The primary oligopeptides arising from the pancreatic digestion (*i.e.* trypsin, chymotrypsin, elastase, CPB and CPA) of *β*-casein were identified, and the probable peptides assessed for the possible hydrolysis points for APN and NEP were assessed manually, *i.e*. Leu-X and Pro-X respectively. Since Leu-pNA was used as the substrate for APN potential P_1 leucine, and its isomer isoleucine, were identified. The estimated concentration of these amino acids was calculated to be 1.5 mM. For NEP internal P_1 proline residues were identified; the concentration was calculated to be 11 mM. Hence, NEP would be functioning at $V_{max.}$

5.3.3 Substrate specificity

There are difficulties in the assay of BBMV oligosaccharidases as individual substrates are hydrolysable by a consortium of enzymes. The oligosaccharidases (SI, MGAM and LPH) work in concert to cleave the glycosidic bonds of carbohydrate oligomers, namely α1,4 linkages (maltase, glucoamylase, sucrase and isomaltase), α1,6 linkages (isomaltase and to a lesser extent glucoamylase), α1,β2 linkages (sucrase), and β1,4 linkages (lactase and phlorizin-hydrolase) (Gray, 2000). Thus the substrates chosen for the characterisation of each enzyme should be specifically cleaved by that enzyme, but in some cases the specific linkage within a carbohydrate is able to be cleaved by a number of BBMV enzymes. Hence, in some cases we were restricted to assaying the activities of a consortium of enzymes, *i.e.* maltase, glucoamylase, sucrase and isomaltase. The concentrations of substrates were, in some cases, limited by the solubility of substrates, but in all cases substrate concentrations were at least five times the suggested K_m.

5.3.4 Reproducibility of brush border enzyme activity

There was no clear pattern to the reproducibility of the kinetic activities of the BB enzymes. For example, while the K_m values were not significantly different between assays for NEP, sucrase and isomaltase their V_{max} values were significantly different. Conversely, some K_m had V_{max} values did not differ between preparations for some enzymes, *i.e.* lactase and glucoamylase, while for others they were significantly different, *i.e.* APN and maltase. In the case of maltolytic activity this may reflect the α1,4 glucolytic activities of a range of enzymes in the oligosaccharidase consortium, *i.e.* maltase, sucrase, isomaltase and glucoamylase (Van Beers et al., 1995b), which display similar specificity (Semenza, 1986), but different kinetics for the hydrolysis of maltose (Ren et al., 2011, Gray et al., 1979). For example, the maltase and glucoamylase sites of MGAM demonstrate K_m and k_{cat} values of 6.17 mM and 47.76 s^{-1}, and 5.53 mM and 21.99 s^{-1}

respectively (Ren et al., 2011). Further, the subunits of SI also differ in their affinity for maltose; sucrase has a K_m and k_{cat} for maltose of 3.6 mM and 12.6 s^{-1} respectively (Gray et al., 1979) while isomaltase has a K_m of 11 mM and a k_{cat} of 11s^{-1} (Gray et al., 1979). The K_m values of these enzymes differed approximately 3 fold so we would expect that their combined hydrolytic contributions to be an amalgamation of these activities. In the case of APN, DP1 has similar specificity (Kozak and Tate, 1982), and may influence the measured kinetic parameters of APN.

5.3.5 The location of the active site on the enzyme

Maltase-glucoamylase and SI are BB enzymes with two domains, each possessing distinct active sites. Both enzymes have a single membrane anchor located at the *N*-terminus of the enzyme. The maltase active site is situated on the MGAM domain closest to the *N*-terminal (adjacent to the membrane) while the glucoamylase site is positioned on the domain towards the *C*-terminal (distal to the membrane) (Sim et al., 2008). The sucrase and isomaltase active sites are located on the *C*-terminal (distal to the membrane) and *N*-terminal domains (adjacent to the membrane) of SI respectively (Semenza, 1986). It is possible that the enzyme with the active site closest to the membrane may exhibit diminished activity due to steric hindrance, but there was no evidence to show that that the position of the active site affected an enzymes hydrolytic activity. For example, sucrase (distal) has a K_m of 40-50 mM and a V_{max} of 400-600 pmol/min for the hydrolysis of sucrose, while isomaltase (proximal) has a K_m of 3 mM and a V_{max} of 800-1,000 pmol/min for the hydrolysis of isomaltotriose. If steric hindrance was a factor then the K_m for isomaltase would be expected to be larger.

5.3.6 Comparison of enzyme activity with published data

The model substrates in these assays were not always the ones previously used. Hence, making it difficult for comparison. Simple precise colorometic assays were needed, and the substrates needed to function for BBMV derived enzymes, and for commercial enzymes chosen as their proxy's. Note that there is very little data that describes enzyme activity as it has been defined here, *i.e.* in pmol/min. Where data was available a comparison of V_{max} has been given. Furthermore, although there is plenty of data on the kinetics of detergent and protease treated BB enzymes there is very little on the kinetics of BBMV-bound enzymes. A table showing the differences in species and form (BBMV-bound, or solubilised, and purified) is given in appendix 1.

5.3.6.1 Neprilysin

It appears that no previous work has been undertaken on BBMV-bound NEP using S-AAPL-*p*NA. However, the hydrolysis of angiotensin 1 by recombinant NEP yields a K_m of 0.055 mM and a k_{cat} of 34.1 s^{-1} (Rice et al., 2004). The K_m for S-AAPL-*p*NA was 1-1.56 mM suggesting that NEP has a greater affinity for angiotensin 1. Since angiotensin 1 is an important component of the Renin-Angiotensin-aldosterone system that regulates plasma sodium levels and blood pressure it is logical that it is a preferred substrate for mammalian enzymes.

5.3.6.2 Aminopeptidase N

No published work was found detailing the kinetics for the hydrolysis of Leu-*p*NA by BBMV APN. However, one study reports the hydrolysis of alanine *p*-nitroanilide (Ala-*p*NA) by BBMV-bound rat APN. These authors determined the K_m for Ala-*p*NA hydrolysis to be 2.33 mM (37°C, pH 7) (Fan et al., 2002), which is 3 to 13 fold greater than the K_m values described here, 0.17 and 0.67 mM (37 ^{O}C, pH 8). This suggests that APN may have a greater affinity for Leu-*p*NA. This was interesting as alanine is thought

to be the preferred substrate for APN (The UniProt Consortium, 2014). However, the difference in assay pH may explain the differences in substrate affinity in this case.

5.3.6.3 Lactase

The K_m values obtained for lactase suggest that the K_m is between 24 and 42 mM. This was in keeping with previous work, which suggest a K_m of 24 mM for rat BBMV-bound lactase (pH 6, 37°C) (Takeuchi et al., 1990). Although others describe a K_m of 16 mM for rat detergent solubilised lactase (pH 6, 37°C) (Mackey et al., 2002), which suggests the presence of the BBMV, and other enzymes affects substrate affinity.

5.3.6.4 Glucoamylase

The substrate most commonly used to assay glucoamylase is soluble starch. This compound is not fully soluble, unless it is gelatinised. Gelatinisation is a process that changes the molecular structure of the starch allowing hydrogen bonding sites on the carbohydrate to engage with water. This process causes thickening of the solution, resulting in a reduction in the diffusive rate of the substrate and enzyme. Hence, this substrate was not used as the model substrate for glucoamylase. Instead pullulan was selected as it readily dissolves in water without the addition of heat, and is colourless. No literature on the hydrolysis of pullulan by rat BBMV-bound glucoamylase was found. One study used soluble starch as a substrate; the K_m was 7.7 mg/ml, (37°C, pH 6) (Takeuchi et al., 1990). Comparing this result with those obtained here we can see that the apparent K_m for pullulan was approximately 4 to 10 fold less than the reported K_m for soluble starch. The size of these substrates may be the cause of their differences in substrate affinity. Soluble starch is a large polymer of linear amylose and branched amylopectin, while pullulan is a polymer of consecutive oligomers of α1,4 linked isomaltotriose linked by α1,6 glucosidic bonds. Since oligosaccharidases are all exo-

enzymes, and are attached to the surface of BBMV, there may be steric hindrance from the membrane vesicles. Also attachment to the BBMV membrane may limit an enzymes access to the reducing ends of the substrate. While the regular structure of pullulan may facilitate its hydrolysis, resulting in less substrate being required to reach half maximum velocity.

5.3.6.5 Sucrase

The activity of sucrase is influenced by the concentration of sodium ions in the aqueous milieu. Hence, in the absence of sodium the K_m for sucrase is 47.5 mM (sucrose, pH 5.9, 37 ^{O}C) (Kolínská and Kraml, 1972), and in the presence of 40 mM of sodium the K_m is 18.5 mM (Kolínská and Kraml, 1972), which suggests that sucrase may have a greater affinity for sucrose in the presence of sodium (Kolínská and Kraml, 1972). The addition of 50 mM of sodium is also reported to cause a decrease in the pH optimum from 6.7 to 5.9 (substrate sucrose) (Kolínská and Kraml, 1972). The reduction in the pH optimum is reported to be caused by deprotonation of an acid group in sucrase in the presence of saturating concentrations of sodium that causes allosteric activation (Vasseur et al., 1982). The buffers used in this work contained sodium in the form of mono-sodium phosphate and disodium phosphate at low concentrations (10 mM), so were unlikely to cause sodium activation. However, this may have been offset by the inclusion of mannitol in the buffering solutions as mannitol is known for maintaining the hydration shell of the enzyme and hence the correct tertiary conformation (Iyer and Ananthanarayan, 2008, Schein, 1990).

Other research reports that rat BBMV-bound sucrase has K_m values of 50.46 mM (sucrose, pH 6.9, 28°C, 20 mM phosphate buffer) (Farooq et al., 2004), 37.9 mM (sucrose, pH 6, 37°C, 100 mM sodium maleate buffer) (Oku et al., 2006), and 34 mM (pH 5.8, 37°C, 100 mM sodium maleate buffer) (Takeuchi et al., 1990). Again, these K_m values

are in keeping with the values obtained here (39-45 mM) even though the buffers contained 10 fold more sodium (Takeuchi et al., 1990, Oku et al., 2006).

5.3.6.6 Isomaltase

The hydrolysis of isomaltotriose by BBMV-bound isomaltase has not been assessed kinetically before, but two studies were found that assessed the hydrolysis of palatinose by rat BBMV-bound isomaltase. The K_m values were 4.5 mM (palatinose, pH 6, 37°C) (Oku et al., 2006) and 5.7 mM (palatinose, pH 5.8, 37°C) (Takeuchi et al., 1990). The K_m values for the hydrolysis of isomaltotriose obtained here were 3.28 and 3.54 mM, so the affinity of isomaltase for isomaltotriose is in keeping with the published K_m for isomaltase.

5.3.6.7 Maltase

Previous reports show that rat BBMV-bound maltase hydrolyses maltose with K_m values of 2.2 mM (37°C, pH 5.8) (Takeuchi et al., 1990) and 4.3 mM (maltose, 37°C, pH 6) (Oku et al., 2006). The K_m values obtained in assay malt2 were consistent with this, *i.e.* a K_m 4.34 mM (maltose, 37°C, pH 6). Again the 2.6 fold difference in the K_m for maltose seen here may reflect the combined hydrolytic activity of all of the α1,4 glucosidic enzymes (Van Beers et al., 1995b).

5.3.7 Differences in enzyme activities due to methodological differences

Brush border digestive enzymes can vary according to circadian rhythms (Saito et al., 1976, Stevenson et al., 1975), nutrient intake (Tanaka et al., 2008) and life stage (Fan et al., 2002). While all efforts were made to standardise the methodology, differences in mucosal sampling and purification techniques can affect results. The pooling of mucosal samples should moderate any large fluctuations in activities, but there will still be some

differences. As to the question as to whether those deviations are acceptable, we used the criterion that enzyme activities should be approximately able to process the inflow of nutrients, and generally they did. Steps were also taken to ensure the methods used in the purification of BBMV remained the same each time. For example, no changes were made to sampling techniques or personnel, and the buffers were kept on ice, the same centrifuges were used to isolate the BBMV, and a cold (-20 °C) Nunc retention plate (ThermoFisher, Auckland, NZ) was used to keep the plate cold during the dispensing of treatments. However, fluctuations in room temperature, timing and handling techniques could vary slightly, which could alter the reaction conditions to some degree, resulting in kinetic differences.

5.3.8 Standardisation for BB *in vitro* digestion

It would be useful to standardise activities against enzyme protein, as is customary, but BBMV incorporate many proteins into their membrane bilayer, including hydrolases, channels and transporter proteins, adhesion proteins, signalling proteins, and immunological proteins (McConnell et al., 2011). It is important therefore to look at how other enzyme admixtures are used for *in vitro* digestion.

Porcine pancreatin is used to imitate exocrine pancreatic secretion used for small intestinal *in vitro* digestions, but it also suffers some of the same complications for standardisation as BBMV. Porcine pancreatin is a secretory product collected from the pancreatic ducts of pigs, which, like BBMV, contains a consortia of digestive enzymes. Pancreatin is used in concentrations that ensure adequate concentrations of the enzymes of interest, normally α-amylase, trypsin or lipase. Since BB enzyme activities are not entirely replicable a similar method of standardisation is suggested here. A good example is lactase - a 40 µl reaction vessel of BBMV (equivalent to 2 mg/ml of mucosal

homogenate) provides 134 -142 pmol/min of lactolysis under the conditions of 10 mM phosphate buffer (divalent cations, 50 mM mannitol) pH 6, 37 °C, 60 minutes.

5.4 Conclusion

The kinetic activities of APN and NEP, and the glycoyltic activity of the oligosaccaridase consortium that were assayed here suggest that the probable enzymatic activities that will be seen *in vitro* could be enough to ensure adequately realistic activities. Although the maximal velocities and substrate affinities of these enzymes were all within the same order of magnitude, there were significant differences in the enzyme activities between BBMV preparations. Despite these differences we cannot preclude the incorporation of the BBMV fraction into a BB phase as yet, as we need to determine whether these enzymes are active in conditions representative of the small intestine.

Although the affinities of many enzymes (*i.e.* lactase, sucrase and isomaltase), for their respective substrates, were in keeping with published results, there were significant, but not large, differences in their maximal velocities. Without purifying BB enzymes to homogeneity, and standardising individual enzymes, it is difficult to assess the hydrolytic rates of individual enzymes. Purifying requisite enzymes would be inconvenient and time consuming, especially if all 106 hydrolases are collected. Further, the isolation of individual enzymes requires BB enzymes to be dissociated from the vesicle, thus altering the normal interaction of nutrients and BB enzymes in their native state, which may have consequences on enzymatic activities. The purification of the BBMV fraction is a relatively short process so is favoured.

From here the next step is to determine whether enzyme activity is altered in the presence of biliopancreatic secretions, and whether biliopancreatic solubilised BB enzymes have different kinetic parameters to their BBMV-bound form.

6 Chapter 6. The effect of biliopancreatic secretions on the activities of brush border enzymes

The detailed kinetic analysis of BB enzyme activities in the previous chapter showed that while the activities of various BB enzymes in BBMV preparations were somewhat different they were of the same order of magnitude. Given that BB enzymes are exposed to biliopancreatic secretions *in vivo* it was then important to determine whether their activities were modified enough in the presence of these secretions to affect the outcomes of *in vitro* analysis.

Bile may change the activities of BB enzymes in two ways; firstly it may bind to the protein component of the enzyme via electrostatic interactions without altering the tertiary structure (Jones, 1992). However, the occupation of all of the requisite binding sites may cause the tertiary structure of a protein to break down, *i.e.* the protein may undergo denaturation (Jones, 1992). Secondly, bile may destabilise the structure of proteins by interfering with the normal organisation of the hydrophobic regions within a protein that confer its tertiary structure (Gass et al., 2007). The denaturation of proteins is a normal process of digestion, which promotes peptidolysis by exposing peptides previously buried within the protein structure (Jones, 1992) to enzymes, such as trypsin and α-chymotrypsin (Gass et al., 2007).

The extent to which protein denaturation occurs depends on whether the head groups of particular bile salts are charged (anionic or cationic) or neutral (non-ionic or zwitterionic) (Otzen, 2011, Jones, 1992). In general, ionic surfactants are more potent denaturing agents than neutral surfactants (Otzen, 2011). The cationic and anionic portions of bile bind to Lys, Arg and His, or Glu and Asp residues respectively (Otzen, 2011). Further, the number

of hydroxyl groups will also affect a bile salts ability to denature proteins, *i.e.* the conjugated dihydroxy bile salt sodium taurodeoxycholate is more potent at disrupting the tertiary structure of protein than the trihydroxy bile salt sodium glycolate (Gass et al., 2007) as it has a larger hydrophobic surface area (Hofmann and Hagey, 2008), *i.e.* it has fewer polar –OH groups.

Brush border enzymes that are associated with the BBMV membrane can be liberated from the membrane by the surfactant action of bile salts, which can disrupt membrane vesicles by dispersing the component lipid bilayers (Nordstrom and Dahlqvist, 1971). In such a situation the solubilised enzyme retains its hydrophobic transmembrane anchor so that the enzyme has an amphipathic nature (Semenza, 1986), *i.e.* both hydrophilic and hydrophobic regions. This property may affect the activities and stabilities of solubilised BBMV enzymes.

Brush border, and other, endogenous enzymes, may all be susceptible to peptidolysis by pancreatic proteases, which have the principal function of reducing dietary protein polymers into short oligomers (Beck, 1973). Pancreatic peptidolyis is often deleterious, but a number of BB enzymes are also activated (undergoing post-translational processing) by pancreatic proteases, *e.g.* SI (Sjöström et al., 1980, Sjöström et al., 1978, Hauri et al., 1979, Zecca et al., 1998). Brush border enzymes may also be liberated from the surface of BBMV by peptidolysis of the stalked region (Semenza, 1986). Such liberation may also be a form of activation as it allows the enzyme the freedom to diffuse into luminal contents. However, there may be physicochemical consequences to losing the transmembrane segment that are yet to be elucidated as stalk proteolysis results in the loss of approximately 3 % of a BB enzymes molecular weight (Semenza, 1986), such as changes in orientation, tertiary confirmation, and solubility. The extent of peptidolysis to other sites of enzymes, particularly at or around the active site is not known, but many BB

enzymes, such as MGAM (Naim et al., 1988b) and SI (Naim et al., 1988a), may be protected by high levels of glycosylation.

Previous works have demonstrated that bile and/or pancreatic enzymes affect both the activities and stabilities of BB enzymes (Seetharam et al., 1976, Maestracci, 1976, Young and Das, 1990); 90 minutes of exposure to human duodenal secretions results in the loss of 70 % of maltase and sucrase activities (Young and Das, 1990). Under the same conditions ALP and lactase are more susceptible to degradation resulting in residual activities of 15 % for ALP, and near complete obliteration of lactase activity (Young and Das, 1990). The deleterious effects of pancreatic enzymes also depend on pH; sucrase activity is not negatively affected by pancreatic enzymes at pH 5, but it loses more than 50 % of activity at pH 8 (Seetharam et al., 1976). Conversely, trehalase maintains 100 % of its activity at pH 8, and maintains less than 25 % of its activity at pH 5 (Seetharam et al., 1976). It should be noted that the pancreatic enzymes also lose activity during aboral transport through the small intestine; amylase, trypsin and lipase are reduced to activities of 74 %, 22 % and 1 % in the ileum (Layer et al., 1986), suggesting that loss of enzyme activity is a normal digestive phenomenon.

The work described in this chapter assesses the extent that bile and pancreatin affect BB enzyme activities using conditions that emulate the normal physiology of the mammalian small intestine..

6.1 Methods

The approach taken was to determine whether biliopancreatic secretions retarded BB enzyme activities within a realistic time frame for *in vitro* analysis, rather than conduct further kinetic analysis of enzyme inhibition. It is, however, acknowledged that subtle inhibitory effects on enzyme activities might be revealed by such detailed kinetic analysis. Two rationales were used to assess the effect of biliopancreatic secretions on BB enzyme activities.

1. **Temporal effects**: The temporal effects of bile and pancreatin on BBMV and BB aqueous enzyme activities (section 6.1.3) were assessed using the rationale based on the physiological ratios of bile, pancreatin and BB enzymes as described in the *in vitro* rationale in section 4.2.

2. **Exposure effects**: The effect of 1 hour of exposure to biliopancreatic components on the activities of BBMV enzyme activities at a single time-point (1 hour) (section 6.1.4) were assessed using the *in vitro* rationale that was adjusted to account for all endogenous and exogenous fluids present in the small intestine (section 4.3).

Since the buffers and assay methods differed between these rationales they will be introduced with the details for each experiment. Further the results will be described separately so they can be compared and contrasted in the discussion.

6.1.1 Reagents

See section 3.1.1.

6.1.2 Brush border enzyme preparations

Small intestinal mucosal scrapings were obtained from eight 10 week old male Sprague Dawley rats. The husbandry and procedures undertaken (AEC 12687) are described in section 3.2. The intestinal scrapings of these rats were used to prepare the BBMV and aqueous fraction based on the calcium precipitation and differential centrifugation method (Boutrou et al., 2008, Kessler et al., 1978, Sakuma et al., 2009) (figure 4), section 3.3.2.

6.1.3 Temporal changes to the activities of BBMV-bound and aqueous BB maltase and ALP following exposure to biliopancreatic components

The aims were to determine whether biliopancreatic secretions affected BBMV-bound and aqueous BB maltase and ALP activities to the same exte. Thus determining whether continued exposure to biliopancreatic secretions altered enzyme activities in the long or short term, and which components of the biliopancreatic/BBMV or BB treatments were responsible for the observed effects. Thus, enzymes were exposed to bile and/or pancreatin for 0, 1 or 2 hours with the objective being to determine whether the BBMV or aqueous fractions would be stable under *in vitro* digestion conditions. The *in vitro* rationale used was based on the ratio of biliopancreatic and BB secretion as detailed in section 4.2.

6.1.3.1 Method for the admixture of BB and biliopancreatic components

The bile and/or pancreatin were added to BBMV and BB aqueous fractions in the ratios and volumes as given in tables 18 and 19. The components of these treatments were either used for assay immediately or they were held at 37 ^{O}C for 1 hour or 2 hours before the substrate was added. This enabled the time at which enzyme activities were modified by bile and pancreatin to be determined. The enzyme admixtures were prepared in 10 ml volumes.

6.1.3.2 Buffers

The buffers used for the temporal study are detailed in section 3.3.3.2.

Table 18. The ratio and concentration of biliopancreatic and BBMV/BB aqueous digestive components used to measure the temporal stability of BBMV/BB aqueous enzyme activities

	Micellar bile preparation	**Pancreatic enzyme preparation**	**BBMV and aqueous preparations**
Initial concentration (mg/ml)	20 (Begley et al., 2005)	1.53 (Ekmekcioglu, 2002)	5[#]
Final concentration (mg/ml)	0.72	0.4	4
Volume (ml)	29 (Kararli, 1995)	208 (Domschke et al., 1977)	572 (Mosenthal, 1911, de Beer et al., 1935)
Ratio	0.04	0.26	0.71

the concentration of the BBMV and aqueous preparations were equivalent to a starting concentration of 5 mg of mucosal scrapings per millilitre buffer.

Table 19. The volumes of components comprising the digestive fluid treatments in a volume of 10 ml, of which 20 µl was used to assay maltolytic and ALP activities.

Fraction	Treatment	BBMV (ml)	BB Aq (ml)	Amylase (ml)	Pancreatin (ml)	Bile (ml)	Buffer (ml)	Total (ml)
BBMV	Buffer	7.1					2.9	10
BB Aq	Buffer		7.1				2.9	10
BBMV	Bile	7.1				0.4	2.5	10
BB Aq	Bile		7.1			0.4	2.5	10
BBMV	Pancreatin	7.1			2.6		0.4	10
BB Aq	Pancreatin		7.1		2.6		0.4	10
BBMV	Pancreatin + bile	7.1			2.6	0.4	0	10
BB Aq	Pancreatin + bile		7.1		2.6	0.4	0	10
Heat tx BBMV	Buffer	7.1					2.9	10
Heat tx BB Aq	Buffer		7.1				2.9	10
Heat tx pancreatin	Buffer				2.6		7.4	10
Buffer	Pancreatin				2.6		7.4	10
Buffer	Bile					0.4	9.6	10
Buffer	Pancreatin and bile				2.6	0.4	7	10
Buffer	Amylase			2.6[#]			7.4	10

The concentration of amylase: 7.39 mg/ml

6.1.3.3 Enzyme assays

Maltase and ALP activities were assayed according to the methods described in sections 3.5.3 and 3.5.4, under the conditions outlined in table 20.

Table 20. Final concentration of substrates, and enzymes and the incubation times of experiments.

Enzyme	Substrate	Substrate concentration (mM)	BBMV/Aqueous (mg/ml)*	Incubation time (min)
Maltase	Maltose	0.73	2	60
ALP	pNP-phosphate	0.67	2	15

*Effective concentration of mucosal extract in final concentration of purified BBMV

6.1.4 The effect of 1 hour of exposure to biliopancreatic components on the activities of BBMV-bound lactase, sucrase, isomaltase, maltase glucoamylase, APN and NEP when diluted by endogenous and exogenous fluid sources

The previous section assessed temporal changes to BB enzyme activities following exposure to biliopancreatic secretions. In contrast the primary aim of this section was to assess the effect of biliopancreatic secretions on the activities, of a wider range of BBMV-bound enzymes, after 1 hour of exposure to bile and/or pancreatin in order to determine whether a second aliquot of the BBMV fraction is warranted half way through an *in vitro* BB digestion.

In vivo digestion is not a sequential process, but for the purpose of *in vitro* simulation sequential digestion is generally accepted (Minekus et al., 2014). However, *in vivo* the process of pancreatic and BB digestion occurs in concert, and together small intestinal transit takes around 4 – 5 hours (DeSesso and Jacobson, 2001, Maqbool et al., 2009, Rao et al., 2009). At present pancreatic *in vitro* digestions typically take 2 hours (Mandalari et al., 2010, Ortega et al., 2011, McDougall et al., 2007, Gil-Izquierdo et al., 2003), so in order to bring the process of small intestinal *in vitro* digestion in line with *in vivo* digestion an adjunct BB *in vitro* digestion would take 2 hours.

This work, undertaken using the *in vitro* digestion based on fluid volume, was described in section 4.3. Hence, the ratios of endogenous and exogenous secretions present in the

small intestine were accounted for, but the absorption of the fluid component of these secretions was not.

In addition to determining whether a second aliquot of BBMV was required for latter *in vitro* digestions, this work aimed to determine whether pancreatic and biliary secretions influenced the activities of BBMV enzymes differently when dilution by all endogenous and exogenous secretions were accounted for. These modifications resulted in changes to the final concentration of biliopancreatic secretions compared to the method section 6.1.3. Hence, the final concentration of biliary secretions increased from 0.72 mg/ml to 1.74 mg/ml, and the final concentration of pancreatic enzymes decreased from 0.39 mg/ml to 0.24 mg/ml. This was due to the starting concentration remaining the same, but the volumes varying. The composition of each reaction are shown in table 21.

Table 21. The ratio, concentrations and volumes of biliopancreatic and BBMV components with dilution by endogenous and exogenous (meal) fluids used to measure the effects on enzyme stability

	Biliopancreatic components				
	Micellar bile preparation	**Pancreatic enzyme preparation**	**BBMV preparations**	**Buffer+**	**Substrate**
Initial concentration (mg/ml)	20 (Begley et al., 2005)	1.53 (Ekmekcioglu, 2002)	33/11#		
Final concentration (mg/ml)	1.74	0.24	6/2*		
Volume (ml)	112 (DeSesso and Jacobson, 2001)	208 (Domschke et al., 1977)	223 (DeSesso and Jacobson, 2001)	431	311
Ratio	0.09	0.16	0.17	0.34	0.24

*: The initial concentration of the BBMV was 33 mg/ml for glucoamylase and NEP and 11 mg/ml for sucrase, isomaltase, maltase, lactase and LAP; # The final concentration of the BBMV was 6mg/ml for glucoamylase and NEP and 2mg/ml for sucrase, isomaltase, maltase, lactase and LAP; + Buffer was added in lieu of digestive secretions contributed by buccal, gastric, and Brunner's glands.

6.1.4.1 Method for the admixture of biliopancreatic and BBMV components

The volumes of bile and/or pancreatin added to the BBMV fraction in the various treatments are given in table 22. These enzyme solutions were mixed and held at 37 ^{O}C for 1 hour before the substrate was added.

Table 22. The volumes of components in the total volume of 40 µl of digestive fluids and substrates to show the effect of biliopancreatic secretions on BBMV enzymes with dilution by endogenous and exogenous fluids.

Fraction	Treatment	BBMV (µl)	Bile (µl)	Pancreatin (µl)	Substrate (µl)	Phosphate buffer (µl)	Total (µl)
BBMV	No treatment	6.9			9.7	23.4	40
BBMV	Pancreatin	6.9		6.5	9.7	23.1	40
BBMV	Bile	6.9	3.5		9.7	19.9	40
BBMV	Pancreatin and bile	6.9	3.5	6.5	9.7	13.4	40
BBMV	No treatment	6.9			0	33.1	40
BBMV	Pancreatin	6.9			0	33.1	40
BBMV	Bile	6.9			0	33.1	40
BBMV	Pancreatin and bile	6.9			0	33.1	40
No enzyme	N/A	0	0	0	9.7	30.3	40

6.1.4.2 Buffers

The buffers used are detailed in section 3.3.3.1

6.1.4.3 Enzyme assays

The methods used for the assay of enzymes after 1 hour of exposure to biliopancreatic secretions are detailed in section 3.5, while the concentrations of substrates in the assays are detailed in table 23. Concentrations of substrates were based on K_m parameters determined in section 5.0.

6.1.5 Analysis

The assays described previously were chosen for their ability to be scaled to a 96 or 384 well format. Absorbances were determined in a SpectroMax Plus (Biostrategy, Auckland, NZ) spectrophotometer with Softmax Pro analysis software 3.0 and Griener GR781101 (384 well) or GR655101 (96 well) transparent microplates (Raylab Ltd). Assays were

undertaken at 37°C in quadruplicate for the assays described in 6.1.3.3 and in sextupicate for 6.1.4.3.

Table 23. Final concentration of substrates, BBMV and the incubation times of experiments.

Enzyme	Substrate	Final concentration of substrate (mM)	Final concentration BBMV (mg/ml)*	Incubation time (min)
Isomaltase	Isomaltotriose	3	2	10
Maltase	Maltose	2	2	10
Sucrase	Sucrose	40	2	30
Lactase	Lactose	30	2	60
Glucoamylase	Pullulan	1mg/ml	6	990
APN	Leu-pNA	0.25	2	60
NEP	S-AAPL-pNA	1	6	360

*Effective concentration of mucosal extract in final concentration of purified BBMV

6.1.6 Statistical analysis

Genstat 17 was used to conduct T-tests, ANOVAs, and post-hoc Bonferroni corrections.

6.1.6.1 Temporal effects:

Data were from a time-course experiment (0 to 120 minutes). Samples are mixed with either brush-border membrane (BBMV), heat-treated (deactivated) BBMV (HBBMV), aqueous BB enzyme or heat-treated aqueous enzyme and either nothing else, bile, pancreatin, or bile and pancreatin. The activities of various enzymes were assessed by colorimetric assay. There were four replicates of each treatment combination, which were repeatedly sampled. The data was analysed using repeated measures ANOVA, post-hoc Bonferroni were used to determine significant differences between treatments. Comparison-wise error rate = 0.0083 across treatment, within time, and 0.0167 across time within treatment.

When comparisons between BBMV and BB aqueous enzyme activities were individual T-tests were performed at each time point.

6.1.6.2 Exposure effects

The effect of 1 hour of exposure to biliopancreatic components on the activities of BBMV-bound lactase, sucrase, isomaltase, maltase glucoamylase, APN and NEP. Data were from endpoint experiments. Samples are mixed with brush-border membrane (BBMV) and either nothing else, bile, pancreatin, or bile and pancreatin. The activity of enzymes was assayed using colorimetic assays. There were 6 replicates of each treatment combination. The data was analysed general ANOVA and post-hoc Bonferroni. Comparison wise error rate 0.0083. A factorial ANOVA was also undertaken on the same data to assess the interactive effect of bile and pancreatin on BB enzyme activities.

6.2 Results

6.2.1 Temporal effects: Comparing the activities of BBMV-bound and aqueous BB maltolytic enzymes at each time point

The activities of maltolytic enzymes were significantly higher in the BBMV fractions (table 24) compared to those in the aqueous fraction at all assay times, *i.e*. at 0 hours (d.f. 1,7, t=3.65, p=0.008), 1 hour (d.f. 1,7, t=3.65, p=0.008) and 2 hours (d.f. 1,7, t=3.63, p=0.008). There were no significant overall differences in the activities of maltase in the BBMV or aqueous fractions when the enzyme was stored at physiological temperature prior to use (37 ^{O}C). These results demonstrate that there was no degradation of maltolytic enzymes over 2 hours in the BBMV or aqueous fractions when the enzyme was stored 37 ^{O}C.

Table 24. Comparison of the activities of BBMV-bound and aqueous maltase (n=4)

Time of enzyme admixture prior to assay		0 hrs	1 hr	2 hrs
Fraction	**Treatment**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**
BBMV	-	80 (3.62)[#a,1]	78(2.42)[a,1]	79 (1.44)[a,1]
Aqueous	-	13.12(0.74)[b,1]	12.71(1.08)[b,1]	12.45(0.38)[b,1]
Heat treated BBMV	-	0	0	0
Heat treated Aqueous	-	0	0	0

#Values in brackets show S.E.M. Activities for maltase between fractions at the same exposure time which have different superscripted letters were significantly different from each other. Activities for BBMV or Aqueous, maltase across time with different superscripted numbers are significantly different from one another (α=0.05).

6.2.2 Temporal effects: Comparing the activities of BBMV-bound and aqueous BB ALP enzymes at each time point

The activities of ALP (table 25) were significantly higher in the BBMV fractions compared with those in the aqueous fraction at each assay time, *i.e.* 0 hours (d.f. 1,7, $t=5.55$, $p<0.001$), 1hour (d.f. 1,7, $t=5.47$, $p<0.001$) and 2 hours (d.f. 1,7, $t=5.41$, $p=0.001$). There were no significant overall differences in the activities of ALP that were stored at physiological temperature (37 ^{O}C). These results demonstrate that there was no degradation of ALP over 2 hours in the BBMV or aqueous fractions at physiological temperature.

Table 25. Comparison of the activities of BBMV-bound and aqueous ALP (n=4)

Time of enzyme admixture prior to assay		0 hrs	1 hr	2 hrs
Enzyme fraction	**Treatment**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**
BBMV	-	317(4.78)$^{\#a,1}$	307(7.13)^{a,1}	304(3.89)^{a,1}
Aqueous	-	113(3.62)^{b,1}	107(2.71)^{b,1}	105(4.10)^{b,1}
Heat treated BBMV	-	0	0	0
Heat treated Aqueous	-	0	0	0

#Values in brackets indicate standard error. Activities for ALP between fractions at the same exposure time which have different superscripted letters were significantly different from each other. Activities for BBMV or Aqueous ALP across time with different superscripted numbers are significantly different from one another (α=0.05).

6.2.3 Temporal effects: BBMV Maltase

6.2.3.1 The effect of biliopancreatic secretions on BBMV maltolytic activities at each exposure time

Immediately following the addition of pancreatin and/or bile (0 hrs) the maltolytic activities in these fractions were not significantly different compared to the BBMV fraction alone (no bile±pancreatin). However, the maltolytic activity seen in the BBMV + bile treatment was significantly greater than that of BBMV + bile and pancreatin suggesting that bile causes an activation of maltolytic enzymes that does not occur when pancreatin is also present, table 26.

After 1 hour of exposure to pancreatin, BBMV maltolytic activity was significantly inhibited compared to the BBMV treatment and the BBMV + bile treatment, but not compared to the BBMV + pancreatin and bile treatment. Again after 2 hours of exposure to pancreatin or bile and pancreatin BBMV maltolytic activity was significantly inhibited compared to BBMV + bile treatment, but not compared to the BBMV treatment. Overall, the maltolytic enzymes associated with BBMV are activated upon exposure to bile alone, but that affect is mitigated over time with the addition of pancreatin.

6.2.3.2 The effect of exposure time to biliopancreatic secretions on the activities of BBMV maltolytic enzymes

Exposure to bile (d.f. 2, 9, F=11.3, p=0.004) or bile and pancreatin (d.f. 2, 9, F=17.71, $p<0.001$) resulted in significant reductions in maltolytic activity over time, table 26. In each case there were significant drops in maltolytic activity from 0 hours to 1 hour of treatment exposures with no further decreases in activities from 1 to 2 hours. In summary, exposure to pancreatin or pancreatin and bile significantly affected the activities of maltolytic enzymes, but the inhibitory action of these compounds does not reach significance until 1 hour of exposure.

Table 26. The activity of BBMV-bound maltase in response to different biliopancreatic treatments over time (n=4)

Exposure to treatment prior to assay		0 hours	1 hour	2 hours
Enzyme fraction	**Treatment**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**
BBMV	Buffer	80.19(3.61)^{a,b}	78.23(2.42)^{a}	78.97(1.44)^{a,b,}
BBMV	Bile	85.57(0.89)^{b,1}	79.47(0.87)^{a,2}	81.14(1.04)^{a,2}
BBMV	Pancreatin	78.27(1.02)^{a,b}	68.48(3.20)^{b}	69.61(3.76)^{b}
BBMV	Pancreatin and bile	76.64(0.72)^{a,1}	70.57(0.37)^{a,b,2}	71.92(0.87)^{b,2}
Buffer	Pancreatin	0	0	0
Buffer	Bile	0	0	0
Buffer	Pancreatin and bile	0	0	0
Heat treated BBMV	Buffer	0	0	0
Heat treated BBMV	Bile	0	0	0
Heat treated BBMV	Pancreatin	0	0	0
Heat treated BBMV	Pancreatin and bile	0	0	0
Buffer	Amylase	1.37	0	1.158

#Values in brackets following activities show S.E.M. Activities within exposure times, across treatment, which have different superscripted letters, and means within treatments, across exposure times, with different superscripted numbers were significantly different from each other as determined by Bonferroni post-hoc analysis. Comparison-wise error rate = 0.0083 across treatment, within time, and 0.0167 across time within treatment.

6.2.4 Temporal effects: Aqueous BB maltase

6.2.4.1 The effect of biliopancreatic secretions on BB aqueous maltolytic activities at each exposure time

Immediately following the addition of pancreatin + bile there was significant augmentation of maltolytic activity in the aqueous phase compared to all other treatments (d.f. 3,11, F=14.12, p<0.001) (0 hrs), table 27. However, bile and pancreatin alone did not significantly alter the activity of BBMV maltolytic enzymes (0 hrs). Following 1 hour

of exposure to pancreatin + bile there was a significant increase in maltolytic activity compared to the aqueous alone and aqueous + bile fraction (d.f. 3,12, $F=9.06$, $p=0.002$). The maltolytic activity in the aqueous fraction was significantly augmented upon exposure to pancreatin and bile at 2 hours compared to all other treatments (d.f. 3, 12, $F=8.48$, $p<0.001$). Hence, there was significant augmentation of maltolytic activity at all time points with the addition of pancreatin and bile at all exposure times. The activities in other treatments were not significantly different to activities in the aqueous fraction.

6.2.4.2 The effect of exposure to biliopancreatic secretions on the activities of BB aqueous maltolytic enzymes

There were no significant differences in the maltolytic activities in the aqueous fraction over time following exposure to bile/and or pancreatin (table 27). This suggests that aqueous maltolytic enzymes are stable in the aqueous fraction.

Table 27. The activity of BB aqueous maltase in response to different biliopancreatic treatments over time (n=4)

Exposure to treatment prior to assay		0 hours	1 hour	2 hours
Fraction	**Treatment**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**
Aqueous	Buffer	13.12(0.74)[#a]	12.71(1.08)[a]	12.45(0.38)[a]
Aqueous	Bile	14.56(0.20) [a]	13.56(0.53)[a]	13.81(0.40)[a]
Aqueous	Pancreatin	14.71(1.80)[a]	14.68(0.56)[a,b]	13.77(1.25)[a]
Aqueous	Pancreatin + bile	18.69(3.07)[b]	17.2(0.26)[b]	19.69(0.63)[b]
Buffer	Pancreatin	0	0	0
Buffer	Bile	0	0	0
Buffer	Pancreatin + bile	0	0	0
Heat treated aqueous	Buffer	0	0	0
Heat treated aqueous	Bile	0	0	0
Heat treated aqueous	Pancreatin	0	0	0
Heat treated aqueous	Bile + pancreatin	0	0	0
Buffer	Amylase	1.37(0.54)	0	1.15(0.05)

#Values in brackets following activities show S.E.M. Activities within exposure times, across treatment that have different superscripted letters, and means within treatments, across exposure times, with different superscripted numbers were significantly different from each other as determined by Bonferroni post-hoc analysis. Comparison-wise error rate = 0.0083 across treatment, within time.

6.2.5 Temporal effects: BBMV ALP

6.2.5.1 The effect of biliopancreatic secretions on BBMV ALP activity at each exposure time

At 0 hours of exposure time the addition of pancreatin or pancreatin and bile to the BBMV fraction significantly inhibited the activities of ALP compared to the treatments BBMV + bile or BBMV alone (d.f. 3,12, $F=13.04$, $p<0.001$), table 28. The same pattern of inhibition was seen at 1 hour (d.f. 3,11, $F=6.83$, $p=0.007$) and 2 hours (d.f. 3,12, $F=18.00$, $p<0.001$) of exposure to these treatments. In contrast to BBMV maltolytic enzymes BBMV ALP activities are inhibited immediately upon exposure to pancreatin containing

treatments and while BBMV maltolytic enzymes are not inhibited by biliopancreatic secretions until 1 hour of exposure.

Table 28. The activities of BBMV-bound ALP in response to different biliopancreatic treatments over time (n=4)

Exposure to treatment prior to assay		0 hours	1 hour	2 hours
Enzyme fraction	**Treatment**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**
BBMV	Buffer	317(4.78)[a]	307(7.13)[a]	304(3.89)[a]
BBMV	Bile	307(5.71)[a]	291(9.52)[a,b]	287 (3.88)[a,b]
BBMV	Pancreatin	269(9.33)[b]	277(4.72)[b]	276(4.69)[b,c]
BBMV	Pancreatin and bile	271(6.45)[b]	271(3.87)[b]	260(5.01)[c]
Buffer	Pancreatin	0	0	0
Buffer	Bile	0	0	0
Buffer	Pancreatin and bile	0	0	0
Heat treated BBMV	Buffer	0	0	0
Heat treated BBMV	Bile	0	0	0
Heat treated BBMV	Pancreatin	0	0	0
Heat treated BBMV	Pancreatin and bile	0	0	0

#Values in brackets following activities show S.E.M. Activities within exposure times, across treatment, that have different superscripted letters, and means within treatments, across exposure times, with different superscripted numbers were significantly different from each other as determined by Bonferroni post-hoc analysis. Comparison-wise error rate = 0.0083 across treatment, within time.

6.2.5.2 The effect of exposure time to biliopancreatic secretions on the activities of BBMV ALP

There were no significant decreases in ALP activities within treatments due to continued exposure to bile and/or pancreatin. The inhibition of BBMV ALP by biliopancreatic secretions occurred immediately, was principally caused by the action of pancreatin, the effect was immediate, and further loss in activity was nominal.

6.2.1 Temporal effects: Aqueous ALP

6.2.1.1 The effect of biliopancreatic secretions on aqueous ALP activity at each exposure time

Upon initial exposure (0 hrs) to pancreatin aqueous ALP activity was significantly inhibited compared to all other fractions (d.f. 3,11, $F=38.24$, $p<0.001$), table 29. Following 1 hour of exposure to bile or pancreatin aqueous ALP activities were significantly decreased compared to the aqueous fraction alone (d.f. 3,12, $F=16.96$, $p<0.001$). After 2 hours of exposure to bile and/or pancreatin aqueous ALP activities were all significantly inhibited (d.f. 3, 12, $F=16.95$, $p<0.001$). However, at all exposure times pancreatin + bile did not significantly inhibit aqueous ALP compared to the aqueous fraction alone. Hence, while exposure to pancreatin significantly inhibited the activity of aqueous ALP at all time points; aqueous ALP was significantly inhibited at only 1 and 2 hours of exposure time, compared to the aqueous fraction alone. This suggests that the inhibitory effect of pancreatin was immediate, but biliary inhibition took longer to take effect.

Table 29. The activity of aqueous BB ALP in response to different biliopancreatic treatments over time (n=4)

Exposure to treatment prior to assay		0 hours	1 hour	2 hours
Fraction	**Treatment**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**	**Mean activity (pmol/min)**
Aqueous	Buffer	113(3.62)[#a]	107(2.71)[a]	105(4.10)[a]
Aqueous	Bile	105(3.52)[a,1]	94.2(1.95)[b,c,2]	88.8(1.76)[b,c,2]
Aqueous	Pancreatin	71.5(2.60)[b,1]	83.6(2.63)[c,2]	80.4(1.57)[c,1,2]
Aqueous	Pancreatin and bile	101(1.25)[a]	102(2.68)[a,b]	96.7(1.81)[a,b]
Pancreatin	Pancreatin	0	0	0
Bile	Bile	0	0	0
Pancreatin and bile	Pancreatin and bile	0	0	0
Heat treated aqueous	Buffer	0	0	0
Heat treated aqueous	Bile	0	0	0
Heat treated aqueous	Pancreatin	0	0	0
Heat treated aqueous	Pancreatin and bile	0	0	0

#Values in brackets following activities show S.E.M. Activities within exposure times, across treatment, that have different superscripted letters, and means within treatments, across exposure times, with different superscripted numbers were significantly different from each other as determined by Bonferroni post-hoc analysis (α=0.05). Comparison-wise error rate = 0.0083 across treatment, within time and 0.0167 across time within treatment.

6.2.1.2 The effect of exposure time to biliopancreatic secretions on the activities of aqueous ALP

Aqueous ALP was significantly inhibited after 1 hour of exposure to bile, and the inhibitory effect remained at 2 hours (d.f. 2, 9, F=10.42, p=0.005). Likewise, when aqueous ALP was exposed to pancreatin there was a significant drop in activity from 0 to 1 hour, which remained at 2 hours (d.f. 2,9, F=7.41, p=0.013). Although exposure to bile or pancreatin alone caused aqueous ALP to be inhibited over time when both were added there was no loss of activity over time suggesting that in concert their inhibitory effects are balanced.

6.2.2 Exposure effects: The effect of 1 hour of exposure to biliopancreatic components on the activities of BBMV-bound lactase, sucrase, isomaltase, maltase glucoamylase, APN and NEP

Biliopancreatic secretions had a significant effect on the activities of BBMV enzymes, figure 23. There were no enzyme activities detected in the negative controls (results not shown).

6.2.2.1 Bile

The addition of bile to the BBMV fraction did not significantly alter the activity of maltase and glucoamylase compared to the BBMV fraction alone. Conversely, the addition of bile significantly inhibited the activities of sucrase (d.f. 3,20, $F=9.12$, $p<0.001$), isomaltase (d.f. 3,20, $F=8.59$, $p<0.001$), lactase (d.f. 3,20, $F=10.54$, $p<0.001$), APN (d.f. 3,20, $F=298.54$, $p<0.001$) and NEP (d.f. 3,20, $F=189.38$, $p<0.001$).

6.2.2.2 Pancreatin

When pancreatin was added to the BBMV fraction it did not significantly change the activities of sucrase or isomaltase, but had a significant negative effect on the activities of lactase (d.f. 3,20, $F=10.54$, $p<0.001$) and APN (d.f. 3,20, $F=298.54$, $p<0.001$), and had a significant positive effect on the activity of NEP (d.f. 3,20, $F=189.38$, $p<0.001$), maltase (d.f. 3,20, $F=120.99$, $p<0.001$) and glucoamylase (d.f. 3,20, $F=9.82$, $p<0.001$).

6.2.2.3 Pancreatin and bile

When both pancreatin and bile were added to the BBMV fraction there was a significant increase in maltase (d.f. 3,20, $F=120.99$, $p<0.001$), glucoamylase (d.f. 3,20, $F=9.82$, $p<0.001$) and NEP activities (d.f. 3,20, $F=189.38$, $p<0.001$) compared to the BBMV fraction alone. Conversely, there was a significant decrease in the activities of isomaltase

(d.f. 3,20, F=8.59, p<0.001), lactase (d.f. 3,20, F=10.54, p<0.001) and APN (d.f. 3,20, F=298.54, p<0.001). While there was no significant change in sucrase activity with the addition of bile + pancreatin.

6.2.3 Exposure effects: The interactive effect of bile and pancreatin on the modification of BB enzyme activity after 1 hour of exposure

Bile and pancreatin can augment, or conversely be deleterious, to the activities of BB enzymes, but when combined they can affect the activities of these enzymes in different ways.

6.2.3.1 Lactase

The addition of pancreatin (d.f. 3,21, F=7.3, *p*=0.014) or bile (d.f. 3,21, F=23.05, *p*<0.001) significantly inhibited the activities of lactase. However, together there was no significant interaction effect, suggesting that their effect on lactase activities was additive.

6.2.3.2 Sucrase

The addition of bile had a significant negative effect on the activity of sucrase (d.f. 3,21, F=22.26, p<0.001). Conversely, pancreatin had a significant positive effect on sucrase activity (d.f. 3,21, F=4.86, p=0.039). Although there was no significant augmentation of sucrase activity with the addition of pancreatin alone. However, when it was added to BBMV in combination with bile, pancreatin appears to rescue some of the activity that was lost when bile was added alone. As the interaction affect was not significant this suggests that the effect of bile + pancreatin is additive.

6.2.3.3 Isomaltase

The addition of bile had a significant negative effect (d.f. 3,21, $F=21.74$, $p<0.001$) on the activity of isomaltase, while pancreatin did not significantly affect its activity. Since the interactive term was not significant it appears that the combined effect of bile + pancreatin is additive.

6.2.3.4 Maltase

The addition of pancreatin had a significant positive effect (d.f. 3,21, $F=361.51$, $p<0.001$) on BB maltolytic activity, while bile had no effect on maltolytic activity. The interaction effect was not significant, which suggests that their combined action is additive.

6.2.3.5 Glucoamylase

Pancreatin had a significant positive effect on the activity of glucoamylolytic enzymes (d.f. 3,21, $F=31.33$, $p<0.001$), while bile did not significantly effect their activity. Again as the there was no significant interactive effect the addition of bile + pancreatin influences glucoamylase in an additive manner.

6.2.3.6 Aminopeptidase N (APN)

The addition of pancreatin (d.f. 3,21, $F=196.63$, $p<0.001$) or bile (d.f. 3,21, $F=698.83$, $p<0.001$) significantly inhibited the activities of APN. Further, when in combination bile and pancreatin acted synergistically, resulting in significantly greater inhibition (d.f. 3,21, $F=24.75$, $p<0.001$).

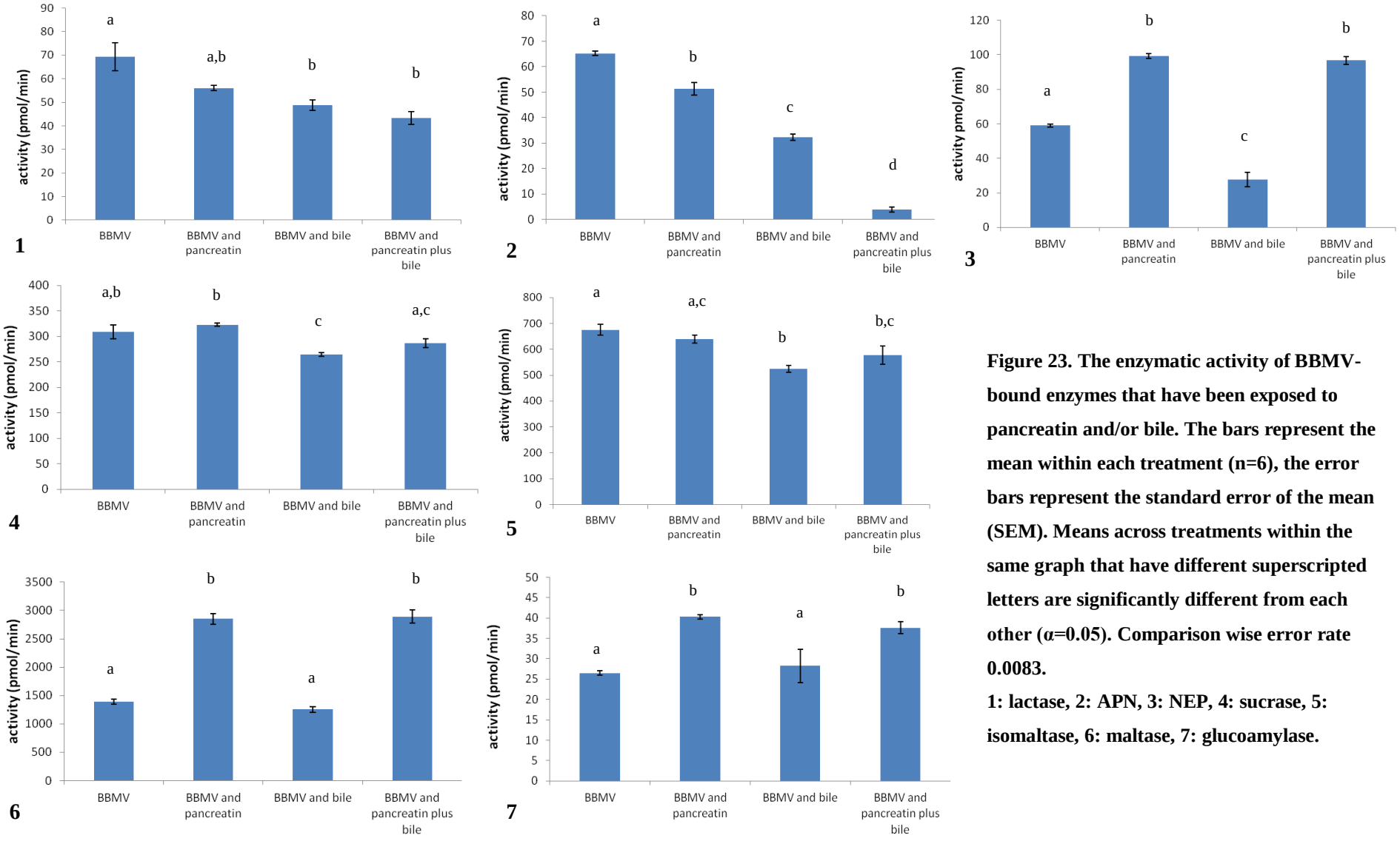

Figure 23. The enzymatic activity of BBMV-bound enzymes that have been exposed to pancreatin and/or bile. The bars represent the mean within each treatment (n=6), the error bars represent the standard error of the mean (SEM). Means across treatments within the same graph that have different superscripted letters are significantly different from each other (α=0.05). Comparison wise error rate 0.0083.

1: lactase, 2: APN, 3: NEP, 4: sucrase, 5: isomaltase, 6: maltase, 7: glucoamylase.

6.2.3.7 Neprilysin (NEP)

The addition of pancreatin had a significant positive effect (d.f. 3,21, $F=487.63$, $p<0.001$) on the hydrolysis of S-AAPL-*p*NA. While the additon of bile has a significant negative effect (d.f. 3,21, $F=46.77$, $p<0.001$). Together bile and pancreatin signifiantly increased NEP activity (d.f. 3,21, $F=24.75$, $p<0.001$). Again, when pancreatin is added NEP recovers some of the activity that is lost with the additon of bile alone suggesting a synergistic model.

6.3 Discussion

6.3.1 Site of BB hydrolysis

By comparing the activities of maltase and ALP in the BBMV-bound and aqueous fractions we can estimate whether the enzymatic activities of maltase and ALP were confined to the surface of BBMV. Maltase and ALP were active and stable in both the aqueous and the BBMV phases. Hence, aqueous enzymes may provide an additional pool of hydrolytic activity that may augment the action of BBMV enzymes *in vivo*. The primary source of aqueous enzymes is from the turnover of small intestinal enterocytes. Enterocytes migrate from the crypts toward the villi tips, before sloughing, lysing and dispersing their contents into the intestinal lumen (Creamer et al., 1961). Murine small intestinal enterocytes migrate from the crypts, and shed from the tips of villi over the course of 2-3 days (Creamer et al., 1961), and the process takes around five days in the human small intestine (Marshman et al., 2002, Van Beers et al., 1995a). Lysed enterocytes contain enzymes originating from cytoplasmic organelles, the cytosol, including active (but immature enzymes), BB enzymes in transit to the apical membrane (Cezard et al., 1979) and cytosolic enzymes with similar specificity to BBMV enzymes *e.g.* cytosolic *β*-glucosidase (EC 3.2.1.21) (The UniProt Consortium, 2015). For the purposes of developing an *in vitro* BBMV phase of digestion, including cytosolic enzymes may be redundant as there is sufficient activity in the BBMV fraction.

6.3.2 Temporal changes to the activities of BB enzymes following prolonged exposure to bile and pancreatin

The activities of BBMV, and aqueous, maltase and ALP differ upon exposure to bile and pancreatin over time. For BBMV-bound maltase there was no significant difference between the treatments at time 0, but by 1 hour there was a significant decrease in maltolytic activity with the addition of bile or bile and pancreatin. The inhibition was

maintained at 2 hours. It is likely that maltolytic enzymes (MGAM and SI) may be protected from inhibition initially by their glycosylation, *i.e.* 30-40 % of MGAM (Naim et al., 1988b, Kelly and Alpers, 1973) and 25 % of SI are carbohydrate residues (Naim et al., 1988a). These high levels of glycosylation are likely to confer resistance to proteolysis (Kingsley et al., 1986, Vaňková et al., 1994). Further, steric hindrance from glycosylated regions close to the stalked region may be why initial exposure (0 hours) to pancreatin does not alter maltolytic activity significantly, *i.e.* it may take longer to cleave the stalked region. As the stalked regions of MGAM and SI are relatively large, 5 nm (Norén et al., 1986) and 3.5 nm (Cowell et al., 1986) respectively, they should be susceptible to proteolytic cleavage (Kenny et al., 1983). Hence, these enzymes should be readily solubilised (Young and Das, 1990).

With increased exposure to bile or pancreatin, only the biliary fractions of BBMV maltase lost activity over time. This suggests that proteolytic solubilisation may not significantly alter the activities of maltolytic enzymes. Further, it appears that sucrase and isomaltase may be more sensitive to biliary inhibition, which may be caused by bile's tendency to bind to and/or denature proteins (Otzen, 2011, Jones, 1992).

In contrast BBMV-bound ALP was significantly inhibited by pancreatin or pancreatin + bile after initial contact (0 hours) with these treatments. However, there were no significant decreases in activities with increased exposure time. Since, ALP is only 12 % glycosylated (Fosset et al., 1974) it seems likely that pancreatic enzymes could have a negative effect on the structural integrity of ALP. Further, as ALP is composed of homo-dimeric sub-units (Fosset et al., 1974) disruption of the BBMV membrane integrity, or the non-covalent interactions that join the dimer, may influence the activity of this enzyme. Indeed, ALP has been found to be inherently unstable following proteolytic treatment (Young and Das, 1990), which suggests that ALP may be subject to proteolytic

degradation as its GPI anchor means that ALP, does not have a stalked segment, and therefore in theory does not undergo proteolytic solubilisation (Young and Das, 1990).

Maltolytic enzymes in the aqueous fraction were affected differently by bile and pancreatin compared to their BBMV-bound counterparts. Exposure to pancreatin + bile resulted in a significant increase in the activities of aqueous maltase at time 0, which were maintained at 2 hours of exposure. This is in keeping with other work that determined that aqueous maltase enzymes are initially activated (undergo an increase in activity compared to BBMV activity alone) upon exposure to pancreatic proteases (Young and Das, 1990) or may be evidence of the post-translational modification (activation) of sucrase (Semenza, 1986). Conversely the addition of pancreatin or bile alone did not significantly alter the activity of maltase compared to BBMV alone at each time point. Increased exposure to bile and/or pancreatin did not change the activities of any of aqueous maltase in any of the fractions over time. This may be evidence that pancreatic proteases are post-translationally modifying immature cytosolic enzymes resulting in active enzymes, *e.g*. pro-SI is enzymatically split into 2 sub-units following exposure to pancreatic proteases resulting in its final form (Semenza, 1986).

In all cases where biliopancreatic inhibition occurs, it develops immediately or by 1 hour with no further loss in activities after 1 hour. This suggests that the inhibitory effect of biliopancreatic secretionsis limited. In the case of bile this may indicate that all potential binding sites on the enzyme may be occupied (Otzen, 2011) resulting in the inhibition of the substrate enzyme interaction or the denaturation of the enzymes in question (Gass et al., 2007).

6.3.3 Comparing the effects of bile and pancreatin on BBMV enzyme activity

The methodological modifications implemented to account for digestive secretions and the meal (section 6.1.4) resulted in differences to the concentration of bile and pancreatin, compared with the method used to assay temporal changes in activities in response to biliopancreatic secretions (section 6.1.3), *i.e.* bile concentration was increased 4.9 fold between the method used for the temporal assessment of activities and the adjusted method which only assessed exposure. However, large differences in enzyme activities were not obtained. Both methods used physiological data drawn from published works to determine the concentration and volume of bile salts added (Begley et al., 2005, DeSesso and Jacobson, 2001, Kararli, 1995). Further, bile was added in a biliary micellar form (Lentle et al., 2012), which is how native BBMV would encounter bile *in vivo* (Hofmann, 1999). In this case it is possible that the inhibitory effects of bile may be limited to bile's ability to bind and/or denature proteins (Jones, 1992). Since no large changes in the activities of the maltolytic enzymes were seen with the addition of a greater concentration of bile, it is possible that all of the potential bile binding sites on BBMV and their requisite enzymes were already occupied, and the increase in bile concentration did not elicit any further denaturing effect.

However, it may also be thought that the pH differences between methods have the potential to cause bile salts to aggregate. The predominant bile salts in mammalian biliary secretions are amidated (with glycine or taurine) (Hofmann and Hagey, 2008) so the majority of bile acids are soluble at physiological pH concentrations (Hofmann and Mysels, 1992) *i.e.* around pH 6 to 8. Since, the pH differences were not large between methods, *i.e.* 6.8 and 6 or 8 respectively, the pH differences are unlikely to cause aggregation.

Calcium salts also have the potential to bind to bile acid anions, with unconjugated bile acids being most susceptible (Hofmann and Mysels, 1992). The binding of calcium to bile salts has the potential to cause aggregation, and therefore the precipitation of bile salts from solution (Hofmann and Mysels, 1992). Calcium was added during the preparation of buffers and BB fractions. The preparation of the BBMV fraction used the calcium precipitation procedure, which involved the addition of calcium chloride (10 mM) for the purpose of aggregating cytosolic and basolateral membranes (Kessler et al., 1978, Schmitz et al., 1973). The pellets were subsequently disposed of following a slow centrifugal spin (3,000 x *g* for 15 min). The majority of added calcium would be associated with the pellet. However, divalent cations were also added to buffers used to dilute and/or assay BBMV enzymes in the exposure assay resulting in the addition of 1 mM of calcium (method 6.1.4). Yet no precipitate was detected which suggests that the critical micellar concentration (mid-point concentration range at which individual bile salts will aggregate), under these conditions, was not reached (Hofmann and Mysels, 1992).

In the exposure assay there was 1.2 fold more pancreatin in the final reaction vessel, *i.e.* 0.24 mg/ml *vs*. 0.197 mg/ml than in the temporal assay. However, in this situation the lower concentration of pancreatin in the temporal assay dramatically inhibited the activities of maltolytic enzymes in this experiment. It could be thought that pancreatic enzymes may be functioning more efficiently at pH 6.8 as this pH is closer to that of pancreatic secretions, *i.e.* pH 7-8 (DeSesso and Jacobson, 2001), but the pH in the duodenum is in constant flux and varies greatly. For example gastric chyme enters the duodenum at a pH between 1 and 5 (Ekmekcioglu, 2002, DeSesso and Jacobson, 2001) and is rapidly buffered by sodium bicarbonate secretions from Brunner's glands (pH 8-8.9), bile (pH 7-8), pancreatin (pH 7.8), and *succus entericus* (7.5-8) (DeSesso and

Jacobson, 2001). As a result luminal duodenal secretions are in the pH range of 5.7-6.4, which increases to approximately 7.4 in the jejunum (Ekmekcioglu, 2002). The pH in the peri-apical space of the proximal jejunum the pH is 6.1 (Lucas, 1983), and the pH optimums of rat MGAM and SI were 6.5 (Flanagan and Forstner, 1978) and 6 respectively (Blair and Tuba, 1963). Hence, under these conditions MGAM and SI activities would be favoured under the temporal and exposure assay conditions respectively. In contrast the 1.2 fold higher concentration of pancreatin seen in the exposure assay resulted in a significant increase in maltolytic activity. This is unusual as pancreatic α-amylase is not thought to hydrolyse maltose (IUBMB, 2013). In the temporal assay pancreatin did not hydrolyse maltose, but α-amylase (positive control) did at a rate 60 fold less than the oligosaccharidases. Pancreatic α-amylase requires a sequence of five α-1,4 glucose units to locate to and it hydrolyses between the second and third glycosidic unit (Quesada-Calvillo et al., 2006) making hydrolysis unlikely. However, other authors showed that trace amounts of glucose are formed during the hydrolysis of starch exposed to pancreatic α-amylase (Bird and Hopkins, 1954), so this requirement may not be absolute. However, this still does not explain the doubling of maltolysis in the exposure assay. It may be that the turnover rate (k_{cat}) has increased (the k_{cat} is determined by the rate at which a substrate diffuses to and binds with it (Fersht, 1999)). Hence, enzymatic solubilisation of the stalked region may improve the diffusion rate of the enzyme resulting in greater maltolytic activity.

In some cases the apparent increase in BBMV enzyme activity may result from pancreatic enzymes augmenting enzyme activity. Hence, the hydrolysis of S-AAPL-*p*NA by NEP was augmented by the action of pancreatic elastase due to mutual substrate specificity (Beck, 1973, Stevens, 2006). Further, pancreatic proteases are unlikely to hydrolyse the stalked region of NEP because the gap between the membrane and stalk is only 2 nm

(Kenny et al., 1983). Hence, BBMV NEP would remain bound to the vesicle. Other enzyme activities that are augmented by pancreatic enzymes were the maltolytic and glucoamylolytic activities of BBMV enzymes. Although the pancreatic hydrolysis of maltose is thought to be minimal (Quesada-Calvillo et al., 2006) due to substrate enzyme requirements (Bird and Hopkins, 1954) it is reasonable to assume that pancreatic α-amylase (The UniProt Consortium, 2014) would be able to hydrolyse pullulan into smaller oligomers by the endohydrolysis of the α-1,4 glucose units.

In all other enzymes pancreatin inhibited BB enzyme activity. This was particularly evident in the case of APN where there was a significant drop in its activity upon exposure to pancreatin. The inhibitive effect was increased with the addition of bile. A possible cause may be the mechanism by which the enzyme is anchored. Aminopeptidase N consists of two monomers that are individually anchored to the membrane by type II anchors (Kenny and Maroux, 1982). It is unlikely that the method of anchor would cause this sort of inhibition as neither SI, MGAM nor NEP were inhibited to the same extent. However, unlike SI and MGAM, APN is composed to two homodimers that are anchored separately and the monomers are held together by non-covalent interactions (Kenny and Maroux, 1982). With a stalked region of 5 nm (Hussain et al., 1981) APN would be readily hydrolysed from the confines of the membrane (Kenny et al., 1983), which may interfere with the non-covalent interactions that keep APN monomers associated. However, APN is thought to be active in a monomeric state (Semenza, 1986) so it seems more likely that bile may be binding to and/or denaturing APN, further work is needed to clarify this.

Enzymes are effected by the presence of bile and pancreatin in different ways. The activity of trypsin and α-chymotrypsin activity are enhanced by bile acids *in vitro* because bile destabilises the tertiary structure of dietary protein (Gass et al., 2007) making

hydrolysis easier. Conversely, the activity of pancreatic α-amylase may be inhibited by bile as it has the propensity to form complexes with amylose and amylopectin, which results in pancreatic α-amylase having limited ability to access the substrate (Takahama and Hirota, 2011). The activity of PLB1 is augmented by bile (Rigtrup et al., 1994) and the hydrolysis of lipids from mixed micelles is improved when proteolytic enzymes hydrolyse and liberate hydrophobic oligopeptides that partition into the oil-water interface (Lueamsaisuk et al., 2013). Hence, it appears likely that similar variations in activities would be seen within the BBMV consortium.

6.4 Conclusion

Bile and pancreatin affect the activities of BBMV-bound and aqueous enzymes in different ways. This may reflect the enzymes individual differences in structure, anchoring, glycosylation, and appropriate binding sites for bile and/or pancreatin. Further examination of the process of enzyme solubilisation is required to determine whether the changes in enzyme activity seen with the addition of bile and pancreatin are driven by changes in the dynamics of the enzymes brought on by solubilisation. Further, although the majority of BBMV enzymes assayed do not lose significant activity over time it is evident that some, like APN, are susceptible to inactivation by biliopancreatic secretions. This is important to note in the development of an *in vitro* BB phase of digestion. Hence, it is advised that a bolus of BBMV enzyme should be added midway through *in vitro* digestion to replenish inactivated enzyme. Further, this will make the system more physiologically relevant as normal digestion involves continuous secretion of digestive hydrolases

7 Chapter 7. A comparison of the kinetics of BBMV-bound and solubilised BB enzymes

In chapter 5 it was demonstrated that BB enzymes from different preparations of BBMV did not have replicable enzyme activities, but the activities were within the same order of magnitude. In chapter 6 it was shown that biliopancreatic secretions significantly affected the activities of BB enzymes, but from the results it was difficult to determine whether the inhibitory effects of bile and pancreatin, on these enzymes, were caused by denaturing, proteolysis or as a consequence of various degrees of enzyme solubilisation from the surface of BBMV. The work in this chapter aims to determine whether enzyme solubilisation was the cause. Hence, this chapter examines the kinetics of a number of these enzymes after their solubilisation by pancreatin or bile and compares these with the kinetic activities of BBMV-bound and cytosolic enzymes

Brush border enzymes that are bound to BBMV can be liberated from the membrane by treatment with bile salts or pancreatic peptidases (Semenza, 1986). Bile salts disrupt the fidelity of BBMV by interfering with the lipid bilayer (Nordstrom and Dahlqvist, 1971) and liberate BB enzymes complete with their hydrophobic transmembrane anchor (Semenza, 1986). These enzymes have 'amphipathic' properties, *i.e.* they have hydrophilic and hydrophobic properties (Semenza, 1986). Conversely, pancreatic peptidases liberate BB enzymes by hydrolysing proteins in the stalked region of BBMV enzymes (Kenny et al., 1983, Semenza, 1986), which dissociates enzymes from their peptide anchors, forming a "soluble" enzyme that is miscible with water (Semenza, 1986, Kenny et al., 1983).

In order to solubilise these enzymes it was important to use physiological concentrations of bile and pancreatic enzymes to disrupt the BBMV membrane, and to cleave the enzyme from the BBMV respectively, in order to emulate what occurs *in vivo*.

The objectives of this work were fourfold, firstly, to determine whether the kinetic activities of enzymes solubilised by bile or pancreatin differed from their bound (BBMV) or soluble cytosolic (aqueous) variants. Secondly, to determine whether pH changes altered the kinetic activities of enzymes from BBMV-bound, soluble, amphipathic, and aqueous fractions. Hence, the activities of each fraction were determined over a range of pH's. Thirdly, it was important to determine whether loss of the hydrophobic transmembrane anchor, on solubilisation with pancreatic peptidases, influenced the kinetic activities of these enzymes. This was done by comparing the activities of the pancreatin solubilised 'soluble' fraction, with the bile solubilised or 'amphipathic' fraction. Finally, the degrees of bile and pancreatic solubilisation were compared with previously reported values.

7.1 Materials and methods

7.1.1 Reagents

Where possible the chemicals purchased were of premium or analytical grade. For a full list see section 3.1.

7.1.2 Animals

Eight 10 week old Sprague Dawley rats were housed in the Food Evaluation Unit, Palmerston North at a temperature of 22±1ºC, humidity of 60±5 %, air exchanged 12 times/hour, and a 12 hour light/dark cycle. For a description of the husbandry and treatments see section 3.2. The animals were euthanased by carbon dioxide inhalation (AEC 12356) at 10 weeks of age, and the small intestinal mucosa harvested.

7.1.3 Brush border enzyme preparations

The small intestinal mucosal scrapings of eight 10 week old Sprague Dawley rats were pooled and the BBMV fraction was prepared from them using a modified version of the calcium precipitation and differential centrifugation method (Boutrou et al., 2008, Kessler et al., 1978, Sakuma et al., 2009) (section 3.3).

7.1.4 Preparation of the enzyme fractions

Four enzyme fractions were used. The vesicle bound enzymes (BBMV fraction), the pancreatin-solubilised enzymes (soluble fraction), bile-solubilised enzymes (amphipathic fraction) and the cytosolic enzymes (aqueous fraction). The aqueous fraction comprised of water miscible enzymes originating in the cytosol of the enterocyte that are likely to be a mixture of the intermediate forms of BB enzymes (Cezard et al., 1979, Maze and Gray, 1980), and BB enzymes that had been mechanically solubilised during the process of purification (Maestracci, 1976). In order to solubilise BBMV anchored enzymes in a relevant way it was necessary to use physiological concentrations and volumes of bile, pancreatic and BBMV secretions present in the small intestine. Wherever possible published volumes and concentrations of bile, pancreatic and BBMV secretions for human subjects were used to determine the relative proportions secreted in the first hour of the post-prandial period by an average 70 kg person. The ratios are given in table 30. The rationale for the concentration and volumes of these secretions are outlined in section 4.2. Other endogenous secretions were not accounted for as they were of low volume and would make little appreciable difference given that the fluid component of digesta would be readily absorbed, *e.g.* water is absorbed from the small intestine of the rat at a rate of 182 µl/cm/hr (Fisher, 1955).

Table 30. The ratio and concentrations of digestive secretions used to assay BB enzymes.

	Micellar bile preparation	Pancreatic enzyme preparation	BBMV and aqueous preparations
Initial concentration (mg/ml)	20 (Begley et al., 2005)	1.53 (Ekmekcioglu, 2002)	4[#]
Final concentration (mg/ml)	0.72	0.39	2
Volume (ml)	29 (Kararli, 1995)	208 (Domschke et al., 1977)	572 (Mosenthal, 1911, de Beer et al., 1935)
Ratio	0.04	0.26	0.71

#relative concentration of mucosal scrapings in homogenate

7.1.5 Preparation of the enzyme fractions

7.1.5.1 BBMV

The BBMV fraction was prepared according to the method outlined in section 3.3. The concentration used was 5 mg/ml (final concentration 2 mg/ml).

7.1.5.2 Aqueous

Supernatant II was retained during BBMV purification as it contained soluble enzymes originating from the cytosol and organelles of small intestinal enterocytes, as well as any soluble (pancreatic enzyme or bile solubilised) BB enzymes entrapped in the mucus overlying the epithelia, and any enzymes that may have been solubilised during the purification process (Maestracci, 1976). This fraction was termed the ‘aqueous fraction’ to differentiate it from the ‘soluble fraction’.

7.1.5.3 Soluble fraction

Pancreatin was used to hydrolyse the stalked region of BB enzymes attached to BBMV. Pancreatin was used as it will simulate the peptidolysis occurring in the small intestine

by pancreatic secretions. Soluble BB enzymes without transmembrane segments were liberated (Semenza, 1986).

Method for preparing pancreatic enzymes (pancreatin)

The volume and concentration of pancreatic secretions was based on the reported quantity of secretin induced pancreatic juices collected from the cannulated pancreatic ducts of human participants (Domschke et al., 1977, Ekmekcioglu, 2002). The rationale is detailed in section 4.2.1.

Preparation of the soluble fraction

The soluble fraction was prepared in batches of 10 mls; comprising of 7.1 ml of 5 mg/ml BBMV, 2.6 ml of 1.53 mg/ml pancreatin and 0.4 ml of buffer 2. The admixture was mixed and stored for 1 hour at 37 ^{O}C before centrifugation at 27,000 x *g*, the supernatant retained as the soluble fraction.

7.1.5.4 Amphipathic fraction

The amphipathic fraction comprised BB enzymes solubilised by bile. This fraction differs from the soluble fraction in that the enzymes maintain their transmembrane anchor, and hence retain their amphipathic properties.

Method for preparing micellar bile

Crude bile salts were reconstituted into a micellar preparation (Lentle et al., 2012) at concentrations and volumes (Begley et al., 2005, DeSesso and Jacobson, 2001), that are commensurate with known volumes of human biliary secretions. The rationale, volume and concentration of bile used, and the method for the preparation of the biliary micellar fraction are detailed in section 4.2.2.

Preparation of the amphipathic fraction

The amphipathic fraction was prepared in volumes of 10 mls; 400 µl of 20 mg/ml micellar preparation was added to 7.1 ml of BBMV and 2.1 ml of buffer 2 and stored for 1 hour at 37 ^{O}C. The fraction was then centrifuged at 27,000 x g and the supernatant retained as the amphipathic fraction.

7.1.6 Enzyme assays

This work assessed the activity of a range of BB enzymes that have different types of membrane anchor. This included one enzyme with a type I peptide anchor (lactase), two with type II peptide anchors (sucrase and maltase) and one with a GPI anchor (ALP). The lengths of the stalked regions of the peptide anchored enzymes, are 2.5 nm for MGAM (Norén et al., 1986) and 3.5 nm for SI (Cowell et al., 1986). No information was found on the effective length of the lactase anchor.

The activities of the oligosaccharidases (maltase, sucrase and lactase) were assayed using a modified version of the glucose-oxidase/peroxidase assay (Dahlqvist, 1964) (section 3.5.3). 20 µl of enzyme and 20 µl of substrate was incubated at 37 ^{O}C for 60 minutes. Then 60ul of GOPOD was added and the sample was incubated at 50 ^{O}C for 20 minutes. Absorbance was read immediately at 510 nm.

Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as a substrate (section 3.5.4). 20 µl of enzyme preparation and 20 µl of substrate were combined and incubated for 15 minutes at 37ºC; the assay was stopped with 40ul 0.1 M NaOH. The absorbance was then read at 405 nm. The concentrations of substrates and enzymes are detailed in table 31.

Table 31. Substrate, enzyme concentrations and assay incubation times for the assay of BBMV, soluble, amphipathic and cytosolic enzymes

Enzyme	Maltase	Sucrase	Lactase	ALP
Substrate	Maltose	Sucrose	Lactose	*p*NP-phosphate
Highest concentration (mM)	58.5	250	250	20
BB enzyme concentration (mg/ml)#	2	2	2	17
Incubation time (min)	60	60	60	15

The final volume of the enzyme fractions, pancreatin, bile, buffer and substrate that are in the final assays are shown in table 32.

Table 32. Enzyme treatments and volumes of digestive secretions to be added to enzyme fraction based on the ratios in table 30.

Fraction	BBMV (µl)	BB Aq (µl)	Pancreatin (µl)	Bile (µl)	Buffer (µl)	Substrate (µl)	Total (µl)
BBMV	14.14				5.86	20	40
Soluble	14.14		5.14		0.72	20	40
Amph*	14.14			0.72	5.14	20	40
Aqueous		14.14			5.86	20	40

*: Amphipathic

7.1.7 Buffers

7.1.7.1 Buffers used in the purification process

Two buffers were used in the isolation of the enzyme fractions (table 33). The pH of the buffer depended on the enzymes being assayed. For the oligosaccharidases (maltase, sucrase and lactase) buffer 1 was a hypotonic 2 mM citric acid/phosphate buffer (Dawson et al., 1986) with 50 mM mannitol (pH 5.6), and buffer 2 was a 10 mM citric acid/phosphate buffer (Dawson et al., 1986) with 50 mM mannitol (pH 5.6). Fractions were then diluted from their original concentration of 33.3 mg/ml to 5 mg/ml with buffer

of the appropriate pH (3.6 – 7.6) and the pH checked, and adjusted, aseptically prior to use using an ISFETCOM S2K922 micropH meter (Total Lab Systems Ltd).

For ALP buffer 1 consisted of a 2 mM Glycine/NaOH buffer (Dawson et al., 1986) with 50 mM mannitol (pH 9.6) and buffer 2 was a 10 mM glycine/NaOH buffer (Dawson et al., 1986) with 50 mM mannitol (pH 9.6). Again aliquots of enzyme were diluted from the original concentration of 33.3 mg/ml to 5 mg/ml with buffer (final concentration after admixture 2 mg/ml) of the appropriate pH (8.6 – 10.6) buffer and the pH checked aseptically prior to use.

Table 33. Buffers used in the preparation of enzyme fractions

Buffer	Enzyme	Buffer*	Concentration (mM)	pH
1	Oligosaccharidases	Citric acid/phosphate	2	5.6
2	Oligosaccharidases	Citric acid/phosphate	10	3.6, 4.6, 5.6, 6.6, 7.6
1	ALP	Glycine/NaOH	2	9.6
2	ALP	Glycine	10	8.6, 9.6, 10.6

7.1.7.2 Buffers used for enzyme assays

The oligosaccharidases were assayed in 10 mM citric acid/phosphate buffer with 50 mM mannitol at pH 3.6, 4.6, 5.6, 6.6, and 7.6 (each in quadruplicate). The assays of ALP were conducted in 10 mM glycine/NaOH buffer with 50 mM mannitol at pH 8.6, 9.6 and 10.6 (each in quadruplicate). Substrates were prepared in buffer 2 of the appropriate pH.

7.1.8 Characterisation of enzyme kinetics and solubilisation

The activities of each of the enzymes were assayed over a series of substrate concentrations. The activities (pmol/min) were then plotted, and the K_m and V_{max} determined by curve fitting. Non-linear curve fitting was conducted in the Origin

graphing programme (OriginLab, Northampton, MA) to fit Hill: y = Vmax *x^n / (k^n+x^n) (the fitted line passes through zero) or Hill 1 curves: y = START + (END - START) * x^n / (k^n + x^n) (where the line did not pass through zero).

7.1.9 Statistical analysis

Data were obtained from kinetic experiments in which enzyme activities were assayed over a series of substrate concentrations (n=4). The data were used to determine the K_m and V_{max} for each enzyme using the Origin graphing program. The values obtained for K_m and V_{max} were compared by Students T-tests. ANOVA could not be used as the K_m and V_{max} values given by Origin are estimates rather than true means. Origin estimates the kinetic parameters using an iterative process, *i.e.* the best fit is obtained by minimising the Chi-squared values of all the parameters.

7.2 Results

7.2.1 Lactase

No lactase activities were found in the soluble fractions at any pH, and there was insufficient lactase activity in the aqueous fractions to enable kinetic characterisation. There were however significant quantities of amphipathic enzyme after solubilisation with bile. The non-linear curve fits for the activities of lactase in the BBMV and amphipathic fractions are shown in figure 24. The kinetic parameters are shown in table 34.

7.2.1.1 The effect of solubilisation on the kinetic parameters of lactase

The only activities that could be compared were the BBMV and amphipathic fractions at pH's 5.6 and 6.6. At pH 5.6 the K_m and V_{max} values were not significantly different from each other, but the non-linear curve fit for the amphipathic fraction at pH 5.6 was poor

(R^2 0.608) due to random variation. At pH 6.6 the K_m for amphipathic lactase was significantly lower than that of the BBMV fraction, *i.e.* 17 *vs.* 27 mM (d.f, 1,50, t=2.3202, p=0.0245) suggesting it had greater affinity for lactose. In contrast to the K_m the maximal velocity in the amphipathic fraction was significantly lower (d.f, 1,50, t=10.6683, p<0.0001) than that of the BBMV fraction at pH 6.6, i.e. 144 vs. 71 pmol/min. Hence, although there was some lactase activity (pH 5.6 and 6.6) in the amphipathic fraction following bile solubilisation there was no lactase activity in the soluble fraction after solubilisation with pancreatin suggesting solubilisation from the surface of BBMV inhibited the activity of lactase.

Table 34. Comparison of the K_m and V_{max} values for brush border lactase in different enzyme fractions at various pH concentrations, obtained from the non-linear curve plots illustrated in figure 24 (n=4).

Fraction	Kinetic parameter	pH 3.6	pH 4.6	pH 5.6	pH 6.6	pH 7.6
BBMV*	K_m (mM)	90 (13.1)^{a}	31(8.8)^{b,c}	23 (4.9)^{b,1}	27 (2.4)^{b,1}	49 (9.8)^{c}
	V_{max} (pmol/min)	177 (9)^{a}	162 (17)^{a,b}	158 (12)^{a,b,1}	144 (3.8)^{b1}	45 (4.9)^{c}
	R^2	0.989	0.964	0.966	0.991	0.880
Soluble#	K_m (mM)	N/D$^{\delta}$	N/D	N/D	N/D	N/D
	V_{max} (pmol/min)	N/D	N/D	N/D	N/D	N/D
	R^2	N/D	N/D	N/D	N/D	N/D
Amph+	K_m (mM)	I/D$^{\gamma}$	I/D	11 (7.5)^{a,1}	17 (3.6)^{a,2}	I/D
	V_{max} (pmol/min)	I/D	I/D	160 (160)^{a,1}	71 (5.7)^{a,2}	I/D
	R^2	I/D	I/D	0.608	0.972	I/D
Aqueous¥	K_m (mM)	I/D	I/D	I/D	I/D	N/D
	V_{max} (pmol/min)	I/D	I/D	I/D	I/D	N/D
	R^2	I/D	I/D	I/D	I/D	N/D

*: BBMV: Brush border membrane vesicle; #: Soluble: soluble enzyme arising from proteolytic solublisation of enzyme from BBMV; +: Amph: amphipathic enzyme arising from BBMV that have been disrupted by bile; ¥: Aqueous: Supernatant II: Enzyme arising from a) the mechanical solubilisation of enzymes, cytosolic contents, and BBMV enzyme that was solubilised by endogenous enzymes prior to sampling. δ, N/D: no data, γ, I/D: insufficient data. Individual t-tests were conducted to determine significant differences between fractions or pH concentrations (α=0.05). The values for K_m or V_{max} within each fraction, and across pH, with different superscripted letters are significantly different from each other. The values for K_m or V_{max} within each pH with different superscripted numbers are significantly different from each other. Values in brackets represent standard error.

7.2.1.2 The effect of pH on the kinetic parameters of lactase in the BBMV and amphipathic fractions

The amphipathic fraction was the only fraction, other than the BBMV fraction, to have sufficient activity to enable enzyme kinetic determination. However, there was only enough data in the amphipathic fraction at pH 5.6 and 6.6 for statistical comparison. This

suggests that amphipathic lactase activity is broadly susceptible to changes in pH. In contrast, the BBMV-bound enzyme was active from pH 3.6 to 7.6.

K_m

The K_m values obtained for BBMV lactase did not vary significantly over the pH range of 4.6 to 6.6 indicating that lactase exhibited broad pH tolerance when bound to BBMV. There were no significant differences between the K_m values at pH 5.6 and 6.6 in the amphipathic fraction.

V_{max}

There were no significant differences in the values of V_{max} obtained for BBMV-bound lactase from pH 3.6 to 5.6. However, there was a slow decline in activities from pH 3.6 to 7.6, with the V_{max} at pH 7.6 BBMV being significantly reduced compared to all other pH concentations, suggesting that the biochemical mileau is important to ensure lactase activity.

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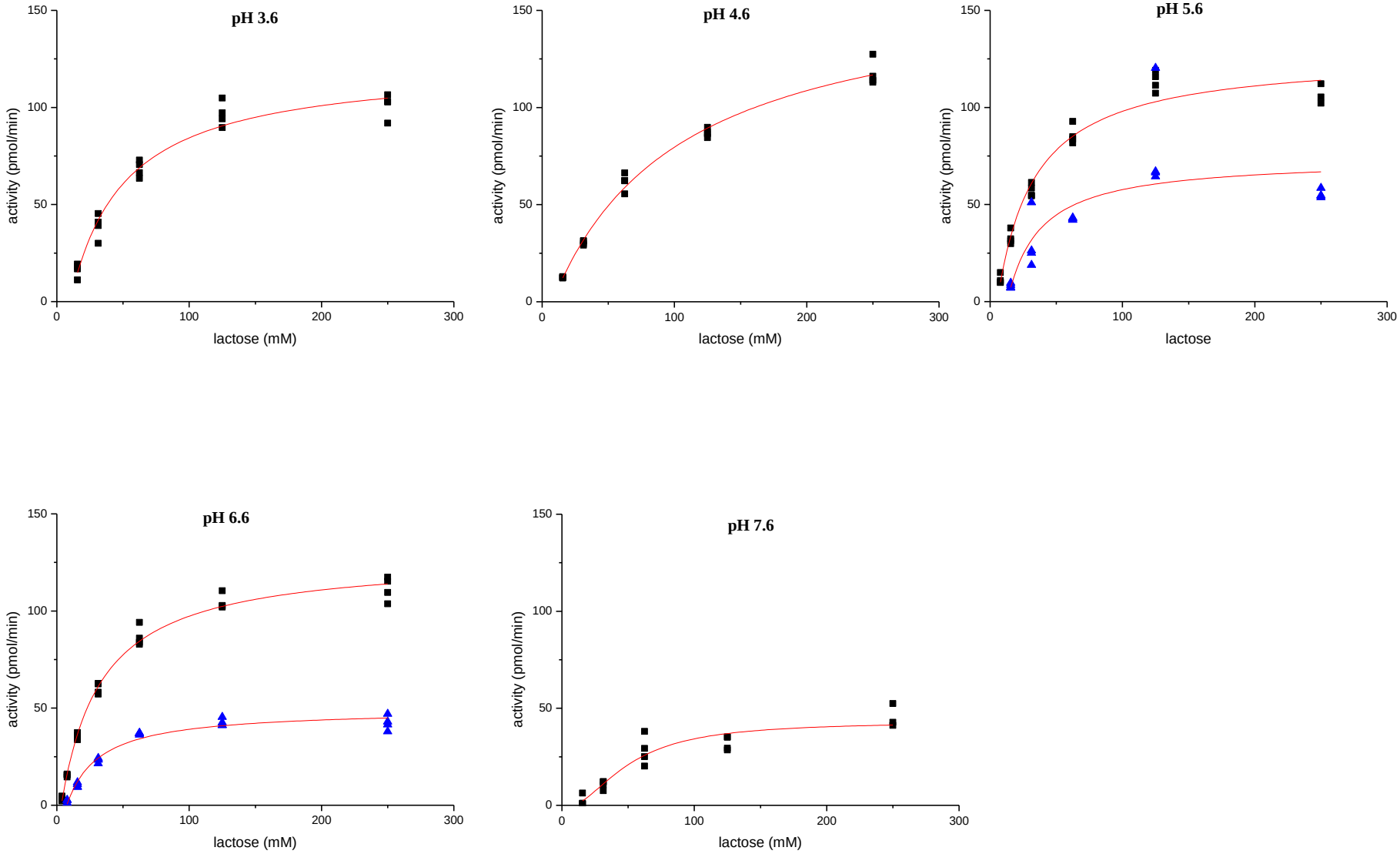

Figure 24. The non-linear curve fits for lactase activity in the BBMV and Amphipathic fractions at 5 pH concentrations. The squares represent the BBMV fraction and the triangles represent the amphipathic fraction (n=4).

7.2.2 Sucrase

Sucrase was active in all four fractions, but activities were low in the soluble and aqueous fractions at pH 7.6 so there was insufficient data to enable characterisation. The non-linear curve fits for sucrase activities are shown in figure 25. The K_m and V_{max} values are shown in table 36.

7.2.2.1 The effect of solubilisation on the kinetic parameters of sucrase

There were no significant differences in the K_m or V_{max} of the soluble, amphipathic or aqueous fractions compared to the BBMV fraction at pH 3.6 that did not continue at higher pH concentrations. However, there was greater retention of sucrase activities in the bile solubilised amphipathic fraction compared to the pancreatin solubilised fraction, at all pH concentrations. This is evident in table 35, which illustrates the ratio of solublised: BBMV-bound sucrase activity; in both fractions there is a lower proportion of activity in the soluble fraction compared to the aqueous fraction at each pH. Solubilisation by bile or pancreatin at any pH resulted in an increase in K_m compared to the BBMV fraction suggesting decreased affinity for the substrate following dissociation from the BBMV. Sucrase activities in the solubilised fractions (bile or pancreatin) did not result in kinetic parameters that were more closely aligned with the aqueous fraction. This suggests that the aqueous fraction is enzymatically distinct from the solubilised fractions, *i.e.* composed of intracellular enzyme variants rather than bile or pancreatin solubilised enzyme.

Table 35. The ratio of solubilised sucrase activities to BBMV-bound activities

	Solubilised activity/BBMV activity				
pH	3.6	4.6	5.6	6.6	7.6
Bile solubilised (amphipathic fraction)	1.022	1.011	0.551	0.753	0.433
Pancreatin solubilised (soluble fraction)	1.040	0.427	0.210	0.061	.

7.2.2.2 The affect pH on the kinetic parameters of sucrase

Sucrase: Km

- BBMV fraction

In general the K_m of the BBMV-bound fraction was not greatly affected by changes in pH suggesting that attachment to the BBMV provides protection from large changes in pH.

- Soluble fraction

The K_m of sucrase varied across pH concentrations. However, there was insufficient activity in the pH 7.6 fraction to assess kinetic parameters suggesting that high pH may influence enzyme/substrate affinity when the enzyme is in a soluble form.

- Amphipathic fraction

The K_m values for sucrase in the amphipathic fraction varied significantly with pH. The K_m at pH 3.6 was greater than those at pH 4.6 - 6.6, but was lower at pH 7.6. In both cases there was increased activities compared to pH 4.6 -6.6 suggesting that amphipathic bile may be more stable at small intestinal pH extremes.

- Aqueous fraction

Although the K_m values of aqueous sucrase do not differ significantly at pH 3.6, 5.6 and 6.6, at pH 4.6 the K_m was 6 fold greater. There was insufficient data to enable kinetic parameters of sucrase in the aqueous fraction to be determined at pH 7.6.

Table 36. Comparison of the K_m and V_{max} values for brush border sucrase in different enzyme fractions and pH concentrations calculated from the non-linear curve fits illustrated in figure 25 (n=4).

Fraction	Kinetic parameter	pH 3.6	pH 4.6	pH 5.6	pH 6.6	pH 7.6
BBMV*	K_m (mM)	55 (17)^{a,1}	15 (2.87)^{b,1}	21 (2.34)^{a,b,1}	22 (3.86)^{a,b,1}	22 (2.03)^{a,b}
	V_{max} (pmol/min)	548 (53)^{a,1}	370 (19)^{b,1}	414 (13)^{b,1}	474 (23)^{a,1}	1031 (27)^{c,1}
	R^2	0.926	0.878	0.961	0.963	0.989
Soluble#	K_m (mM)	72 (21)^{a,1}	67 (34)^{a,1,2}	23 (8.5)^{b,1,2}	86 (39)^{a,1,2}	I/Dγ
	V_{max} (pmol/min)	570 (55)^{a,1}	158 (26)^{b,2}	87 (14)^{c,2}	129 (19)^{b,c,2}	I/D
	R^2	0.937	0.828	0.948	0.903	I/D
Amph+	K_m (mM)	64 (20)^{a,b,c,1}	59 (6.9)^{b,2}	30 (1.37)^{c,2}	85 (22)^{a,2}	19 (2.96)^{d}
	V_{max} (pmol/min)	560 (57)^{a,1}	374 (14)^{b,1}	228 (3)^{c,3}	357 (30)^{b,3}	446 (26)^{a,2}
	R^2	0.924	0.986	0.998	0.965	0.985
Aqueous¥	K_m (mM)	63 (20)^{a,1}	378 (134)^{b,3}	59 (9.3)^{a,3}	71 (19.)^{a,2}	I/D
	V_{max} (pmol/min)	578 (59)^{a,1}	340 (93)^{b,1}	69(4)^{c,2}	133 (11)^{d,2}	I/D
	R^2	0.923	0.976	0.930	0.963	I/D

*: BBMV: Brush border membrane vesicle; #: Soluble: soluble enzyme arising from proteolytic solublisation of enzyme from BBMV; +: Amph: amphipathic enzyme arising from BBMV that have been disrupted by bile; ¥: Aqueous: Supernatant II: Enzyme arising from a) the mechanical solubilisation of enzymes, cytosolic contents, and BBMV enzyme that was solubilised by endogenous enzymes prior to sampling. γ, I/D: insufficient data. Individual t-tests were conducted to determine significant differences between fractions or pH concentrations (α=0.05). The values for Km or Vmax within each fraction, and across pH, with different superscripted letters are significantly different from each other. The values for K_m or V_{max} within each pH with different superscripted numbers are significantly different from each other. Values in brackets represent standard error.

Sucrase: V_{max}

- BBMV

The maximal velocity of sucrase in the BBMV fraction was at pH 7.6 mM suggesting attachment to the vesicle confers a benefit to activity at higher pHs.

- Soluble

The V_{max} for sucrase in the soluble fraction was pH 3.6. Values dropped significantly with increases in pH until there was insufficient data to determine kinetic parameters at pH 7.6. Suggesting that once sucrase is solubilised by proteases it may function better at lower pH concentrations.

- Amphipathic

The V_{max} values for the amphipathic fraction varied significantly with pH. The maximal activities of sucrase were significantly increased at the extremes in pH, *i.e.* pH 3.6 and 7.6. However, the increase in activity at 7.6 most resembles the increase in activity seen by BBMV-bound sucrase at 7.6 suggesting that retaining the transmembrane segment may help maintain sucrase's structural attributes.

- Aqueous fraction

The maximal velocity of sucrase in the aqueous fraction was pH 3.6, but activities decreased with increases in pH. This trend in activity resembles the pattern seen in the soluble fraction, particularly the low results of 87 and 69 pmol/min at pH 5.6. The pH most similar to the pH optimum.

The impact of pH on solubilised enzyme are shown in table 35. It is evident that, comparative to the BBMV fraction, enzyme solubilised sucrase loses activity with each increase in pH, *i.e.* the proportion of active enzyme reduces with each increase in pH. With pancreatin solubilisation being most deleterious.

7.2.3 Maltase

The non-linear curve fits for maltase activity are shown in figure 26. The kinetic parameters are shown in table 38.

7.2.3.1 The effect of enzyme solubilisation on the kinetic parameters of maltase

In general the solubilisation of BBMV maltase by bile or pancreatin resulted in significant increases in the K_m. Solubilisation with bile had little effect on maltase activities in the amphipathic fraction. While solubilisation with pancreatin resulted in significant decreases in maltase activities in the soluble fraction.

The ratio of solubilised (bile or pancreatin) to BBMV-bound maltase activities are shown in table 37. From pH 4.6 – 7.6 the ratio of bile solubilised maltase activities to BBMV-bound maltase activities are close to 1 suggesting that maltase activity was not significantly affected by bile solubilisation or moderated changes in pH. However, at pH 3.6 the ratio was 0.58 suggesting that low pH concentrations negatively influenced maltase activities. Conversely, the ratio of pancreatin solubilised maltase to BBMV-bound maltase activities are ~ 0.6 suggesting that solubilisation from the BBMV negatively influences activity. The ratio of pancreatin solubilised maltase to BBMV-bound maltase is 0.06 at pH 3.6 suggesting that maltolytic activity is negatively influenced by low pH.

Table 37. The ratio of solubilised maltase activities to BBMV-bound activities

	Solubilised activity/BBMV activity				
pH	3.6	4.6	5.6	6.6	7.6
Bile solubilised (amphipathic fraction)	0.5836	1.0610	0.9827	0.9458	0.9727
Pancreatin solubilised (soluble fraction)	0.0611	0.6549	0.6135	0.5729	0.6092

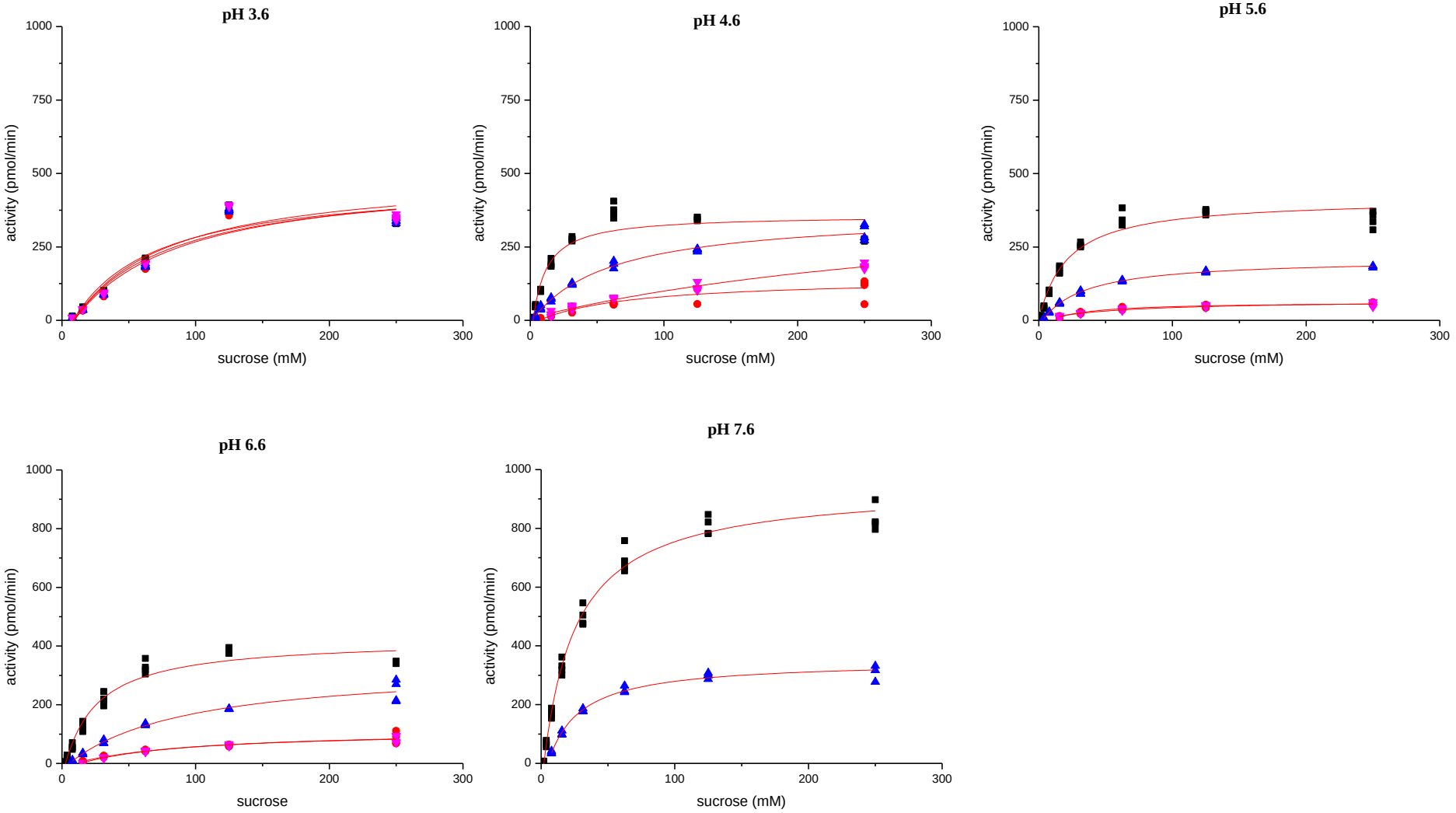

Figure 25. The non-linear curve fits for the activity of sucrase in the various fractions. The black data points represent BBMV activity over different substrate concentrations; the red data points represent soluble enzyme activity; the blue data points represent amphipathic enzyme activity; the pink data points represent aqueous enzyme activity (n=4).

7.2.3.2 The effect of pH on the kinetic activities of maltase

Maltase: Km

- BBMV fraction

The K_m values for the BBMV-bound maltolytic enzymes were not significantly different over the pH concentrations of 4.6 to 7.6, but it was significantly increased at the lower pH of 3.6 suggesting lower affinity for substrate at a low pH.

- Soluble fraction

The K_m values for the soluble fraction were not greatly affected by changes in pH from 4.6-7.6. However, there was a significant decrease in the K_m at pH 3.6. This may signify that there may be tighter binding to the maltose, without significant hydrolysis.

- Amphipathic fraction

The K_m values of the aqueous fraction were not significantly affected by changes in pH from pH 4.6-7.6. However, there was a significant increase in the K_m at pH 3.6, not unlike that seen in the BBMV fraction suggesting that the maltolytic enzymes, in amphipathic fractions were acting similarly to those in the BBMV-fraction.

- Aqueous fraction

There was a trend towards an increase in K_m with successive increases in pH from 3.6 to 7.6. The lowest value for K_m was at pH 3.6. Aqueous maltolytic enzymes appear to behave in a similar way to maltolytic enzymes in the soluble fraction, which may indicate the source of some of the component enzyme, *i.e*. a portion of maltolytic enzymes in the aqueous phase may be have been solubilsed *in vivo,* and collected with mucosal scrapings.

Table 38. Comparison of the K_m and V_{max} values for brush border maltase in different enzyme fractions and pH concentrations calculated from the non-linear curve fits illustrated in figure 26 (n=4)

Fraction	Kinetic parameter	pH 3.6	pH 4.6	pH 5.6	pH 6.6	pH 7.6
BBMV[1]	K_m (mM)	0.93 (0.15)^{a,1}	0.36 (0.02)^{b,1}	0.27 (0.10)^{b,1}	0.40 (0.10)^{b,1}	0.29 (0.07)^{b,1}
	V_{max} (pmol/min)	622 (30)^{a,1}	623 (35)^{a,1}	577 (68)^{a,1}	590 (44)^{a,1}	586 (43)^{a,1}
	R^2	0.971	0.954	0.855	0.922	0.936
Soluble[2]	K_m (mM)	1.41 (1.04)^{a,1,3}	4.63 (0.30)^{b,2}	4.17 (0.42)^{b,2}	5.28 (0.37)^{b,c2}	5.1 (0.05)^{c,2}
	V_{max} (pmol/min)	38 (5)^{a,2}	408 (8)^{b,2}	354 (10)^{b,2}	338 (7)^{b,2}	357 (10)^{b,2}
	R^2	0.17	0.994	0.983	0.983	0.985
Amph[3]	K_m (mM)	3.9 (0.28)^{a,2}	0.52 (0.14)^{b,1}	0.61 (0.14)^{b,3}	0.84 (0.14)^{b,c,3}	1.07 (0.04)^{c,3}
	V_{max} (pmol/min)	363(8)^{a,3}	661 (77)^{b,1}	567 (43)^{b,1}	558 (29)^{b,1}	570 (35)^{b,1}
	R^2	0.993	0.921	0.916	0.951	0.942
Aqueous[4]	K_m (mM)	1.66 (0.33)^{a,3}	2.3 (0.33)^{a,b,3}	2.19 (0.31)^{a,b,4}	2.72 (0.21)^{b,4}	2.72 (0.23)^{b,4}
	V_{max} (pmol/min)	280 (19)^{a,4}	499 (21)^{b,c,3}	498 (20)^{b,c,1}	463 (10)^{b,3}	517 (12)^{c,1}
	R^2	0.956	0.968	0.968	0.990	0.989

*: BBMV: Brush border membrane vesicle; #: Soluble: soluble enzyme arising from proteolytic solublisation of enzyme from BBMV; +: Amph: amphipathic enzyme arising from BBMV that have been disrupted by bile; ¥: Aqueous: Supernatant II: Enzyme arising from a) the mechanical solubilisation of enzymes, cytosolic contents, and BBMV enzyme that was solubilised by endogenous enzymes prior to sampling. γ, I/D: insufficient data. Individual t-tests were conducted to determine significant differences between fractions or pH concentrations (α=0.05). The values for Km or Vmax within each fraction, and across pH, with different superscripted letters are significantly different from each other. The values for Km or Vmax within each pH with different superscripted numbers are significantly different from each other. Values in brackets represent standard error.

Maltase: V_{max}

- BBMV fraction

The maximal velocities of maltase did not vary with pH over the range of 3.6 to 7.6.

- Soluble fraction

The maximal velocity of maltase in the soluble fraction did not vary significantly over the pH range of 4.6-7.6. However, the V_{max} at pH 3.6 was significantly lower.

- Amphipathic fraction

The maximal velocity of maltase in the amphipathic fraction was significantly lower at pH 3.6, but did not vary significantly over the range of 4.6 to 7.6.

- Aqueous fraction

There was a trend towards an increase in maximal velocities with increases in pH. The lowest V_{max} was seen at pH 3.6, which was similar to the pattern that was seen in the soluble and amphipathic fraction.

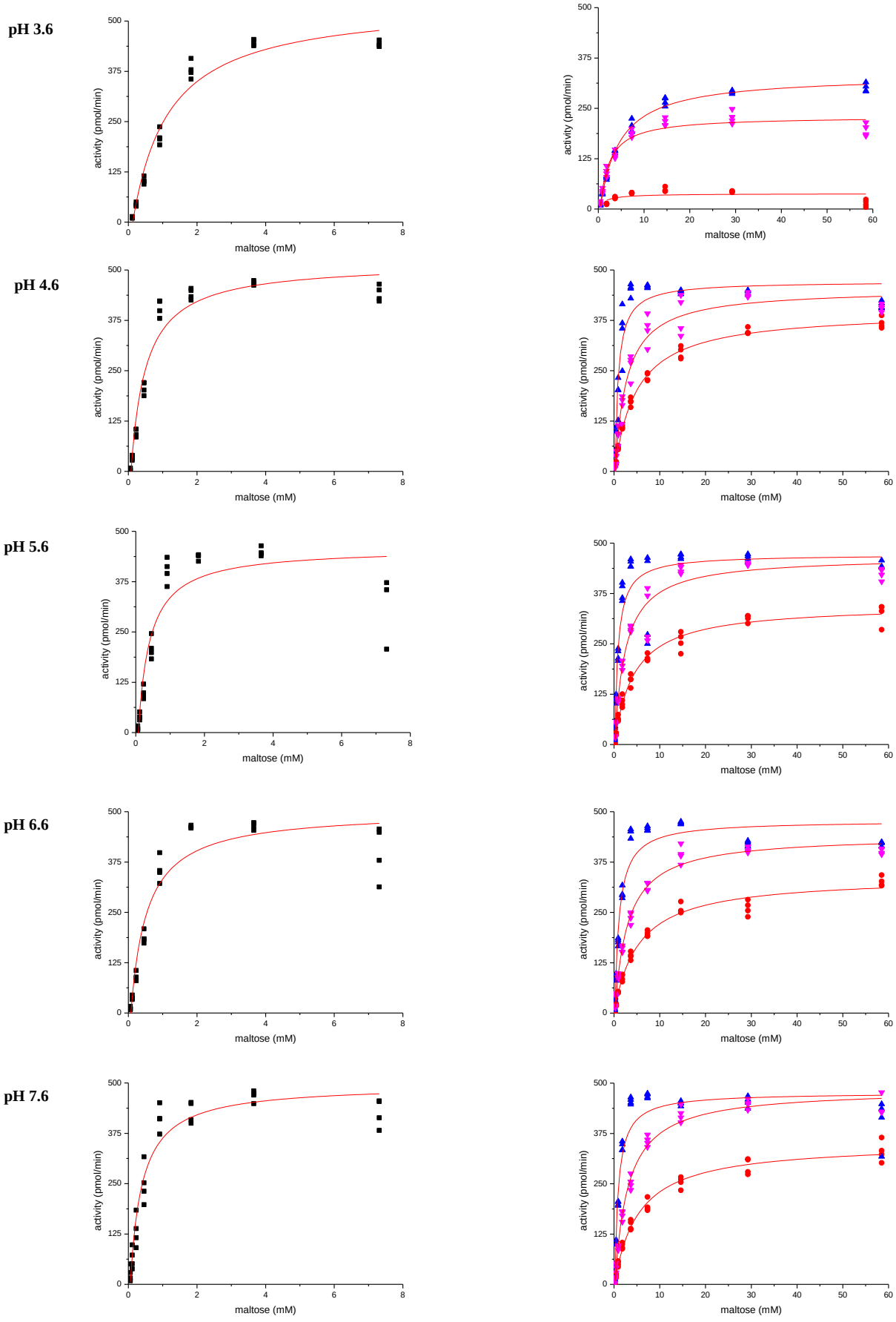

Figure 26. The non-linear curve fits for maltase activity in different enzyme fractions and at different pH concentrations. The black data points represent BBMV activity over different substrate concentrations; the red data points represent soluble enzyme activity; the blue data points represent amphipathic enzyme activity; the pink data points represent aqueous enzyme activity(n=4).

7.2.4 Alkaline phosphatase

The non-linear curve fits for ALP activities (pmol/min) are shown in figure 27. The kinetic parameters are shown in table 40.

7.2.4.1 The effect of enzyme solubilisation on the kinetic parameters of ALP

Solubilisation by bile or pancreatin had a significant negative effect on the enzymatic activities of ALP, *i.e.* the V_{max} values were significantly lower, up to 10 fold less, following solubilisation by bile or pancreatin. Further, pancreatin solubilisation caused a significantly greater losses in the maximal velocities of ALP compared to BBMV-bound ALP than bile solubilisation. Hence, the ratios of solubilised to BBMV-bound ALP activities are particularly low, table 39.

Table 39. The ratio of solubilised ALP activities to BBMV-bound activities

Solubilised activity/BBMV activity			
pH	**8.6**	**9.6**	**10.6**
Bile solubilised (amphipathic fraction)	0.1149	0.0993	.
Pancreatin solubilised (soluble fraction)	0.2299	0.2085	0.2634

7.2.4.2 The effect of pH on the kinetic parameters of ALP

Km

- BBMV fraction

The K_m was lowest at pH 9.6 (0.41 mM) and was increased at the pH 8.6 and 10.6, *i.e.* 0.82 and 1.75 mM respectively. Suggesting that the K_m for ALP was influenced by changes in pH.

- Soluble fraction

The K_m for ALP in the soluble fraction was decreased from pH 8.6 to 9.6, *i.e*. 3.88 mM and 1.09 mM respectively. There was no enzyme activity detected in the soluble fraction at pH 10.6 suggesting that ALP activity is inhibited at high pH concentrations.

- Amphipathic fraction

The K_m for ALP in the amphipathic fraction was significantly lower at pH 8.6 than at pH 9.6 or 10.6. The K_m values at pH 9.6 and 10.6 were not significantly different from one another. At pH 10.6, amphipathic ALP was still active, which suggests that, comparatively speaking, the amphipathic form of ALP is more stable than the soluble form of ALP.

- Aqueous fraction

There were no significant differences in the K_m values for ALP in the aqueous fraction at pH 8.6 and 9.6, but the K_m was significantly higher at pH 10.6.

Table 40. Comparison of the K_m and V_{max} values for brush border alkaline phosphatase in different enzyme fractions and pH concentrations calculated from the non-linear curve fits illustrated in figure 27 (n=4).

Fraction	Kinetic parameter	pH 8.6	pH 9.6	pH 10.6
BBMV[1]	K_m (mM)	0.82 (0.02)[a,1]	0.41 (0.04)[b,1]	1.75 (0.24)[c,1]
	V_{max} (pmol/min)	1566 (19)[a,1]	1218 (32)[b,1]	1025 (59)[c,1]
	R^2	0.996	0.973	0.963
Soluble[2]	K_m (mM)	3.88 (0.86)[a,2]	1.09 (0.11)[b,2]	N/D
	V_{max} (pmol/min)	180 (21)[a,2]	121 (5)[b,2]	N/D
	R^2	0.944	0.967	N/D
Amph[3]	K_m (mM)	0.58 (0.05)[a,3]	0.94 (0.07)[b,2]	1.13 (0.08)[b,2]
	V_{max} (pmol/min)	360 (9)[a,3]	254 (7)[b,3]	270 (7)[b,2]
	R^2	0.978	0.98	0.984
Aqueous[4]	K_m (mM)	0.537 (0.15)[a,1,3]	0.46 (0.04)[a,1]	2.21 (0.74)[b,1,2]
	V_{max} (pmol/min)	471 (40)[a,4]	362 (10)[b,4]	171 (25)[c,3]
	R^2	0.747	0.969	0.807

*: BBMV: Brush border membrane vesicle; #: Soluble: soluble enzyme arising from proteolytic solublisation of enzyme from BBMV; +: Amph: amphipathic enzyme arising from BBMV that have been disrupted by bile; ¥: Aqueous: Supernatant II: Enzyme arising from a) the mechanical solubilisation of enzymes, cytosolic contents, and BBMV enzyme that was solubilised by endogenous enzymes prior to sampling. γ, I/D: insufficient data. Individual t-tests were conducted to determine significant differences between fractions or pH concentrations (α=0.05). The values for Km or Vmax within each fraction, and across pH, with different superscripted letters are significantly different from each other. The values for Km or Vmax within each pH with different superscripted numbers are significantly different from each other. Values in brackets represent standard error.

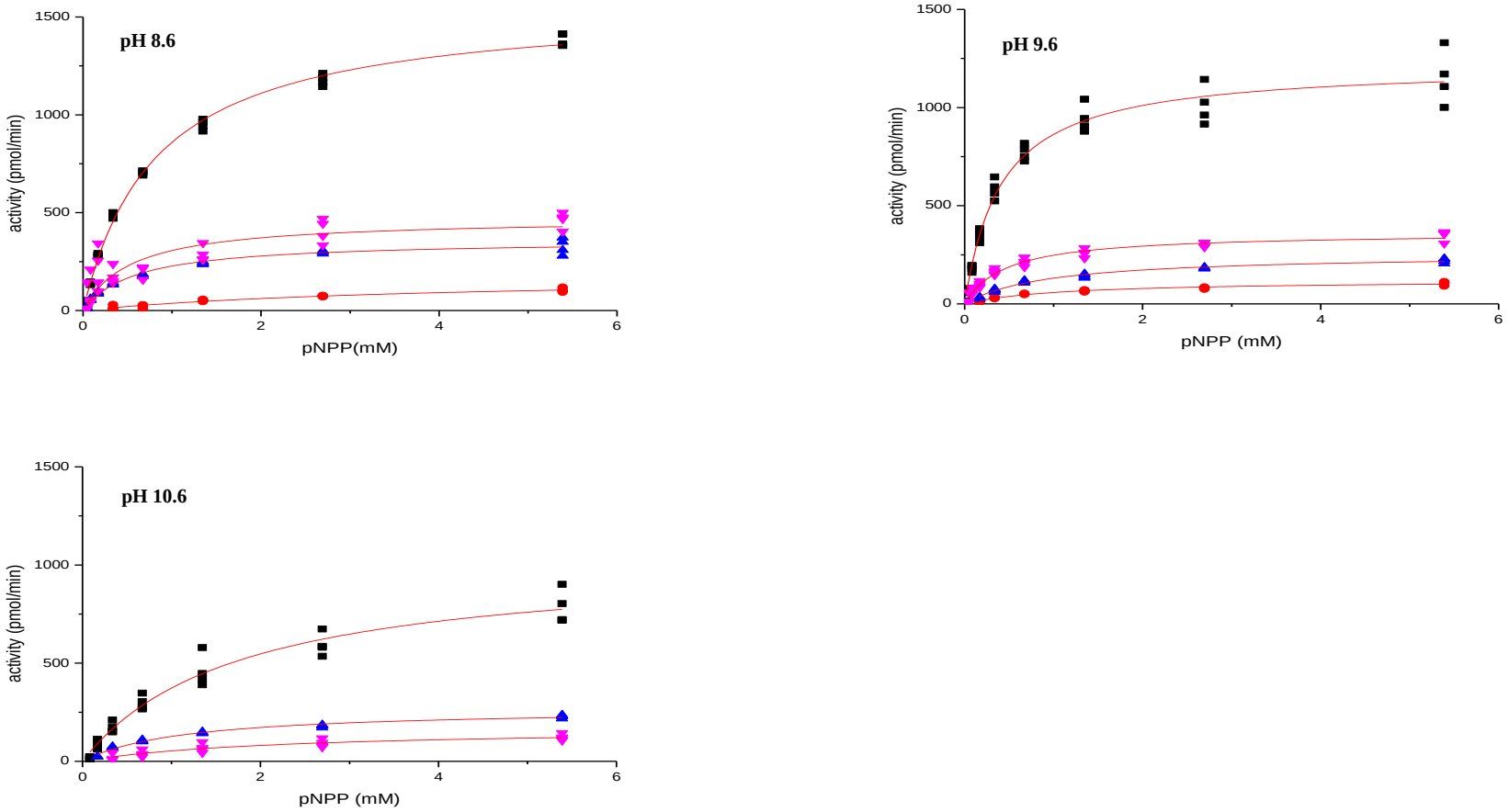

Figure 27. The non-linear curve fits for alkaline phosphatase activity in different enzyme fractions and at different pH concentrations The black data points represent BBMV activity over different substrate concentrations; the red data points represent soluble enzyme activity; the blue data points represent amphipathic enzyme activity; the pink data points represent aqueous enzyme activity (n=4).

$\underline{V_{max}}$

- BBMV fraction

There were significant drops in the V_{max} for ALP in the BBMV fraction with increasing pH. Suggesting inherent sensitivity to pH.

- Soluble fraction

In the soluble fraction the V_{max} for ALP significantly decreased between pH 8.6 and 9.6. There was no ALP activity in the soluble fraction at pH 10.6 suggesting that soluble ALP activity is destroyed at high pH concentrations.

- Amphipathic fraction

In the amphipathic fraction there was a significant reduction in the V_{max} of ALP with the increase in pH from 8.6 to 9.6. The significant decrease in activity was maintained at pH 10.6

- Aqueous

There were significant decreases in aqueous ALP activities with increasing pH.

7.3 Discussion

7.3.1 Solubilisation of enzymes by bile and pancreatin

Enzyme solubilisation has the potential to promote greater BB enzyme activities due to improved enzyme diffusivity following solubilisation because the rate at which a particle (or enzyme) diffuses in solution is inversely proportional to its size (Cu and Saltzman, 2009, Norris et al., 1998). Upon liberation from the BBMV BB enzymes should more readily diffuse into luminal contents. Hence, the rate at which intact BBMV diffuses in the peri-apical and luminal space would be reduced compared with that of soluble BB enzymes, particularly in the presence of viscous digesta. The ability of the enzyme to freely diffuse could, on the outside, appear to benefit the activity of a particular enzyme.

However, results indicated that there were decreases in the maximal velocities of some enzymes following solubilisation. However, it must be noted that this work did not assess the residual activity in the pellets following solubilisation by bile or pancreatin. In retrospect this would have been useful. Hence, for some enzymes, the BBMV may retain the active enzyme. However, it could also be thought that those enzymes were inactivated, this notion will be investigated later in the discussion.

Another point of interest is that the kinetic activities of solubilised enzymes, in general, did not approximate activity in the aqueous fraction, suggesting that aqueous enzymes were of different origin.

7.3.1.1 The effect of attachment to BBMV on the kinetic parameters of lactase.

The stalked region of lactase appears to be important to its ongoing activity. Based on previous work (Young and Das, 1990) 60 % of lactase activity was expected to be present in the soluble fraction following treatment of BBMV with pancreatic enzymes. However, there was no activity suggesting that cleavage of the stalked region, and separation from the BBMV, resulted in a catastrophic loss of lactase activities. However, since no information on the stalk length of lactase was found it may be that lactase is not susceptible to stalk proteolysis.

7.3.1.2 The effect of attachment to BBMV on the kinetic parameters of sucrase

Retaining the transmembrane segment, following solubilisation from BBMV, appears to have a significant influence on the kinetic parameters of sucrase, but this effect varies with pH. The ratio of soluble to amphipathic activities (V_{max}) gives an indication as to whether the stalked region is important to ongoing activity. At pH 3.6 the ratio is 1.018, suggesting that proteolysis of the stalk may not adversely affect sucrase activity.

However, at pH 4.6, 5.6, 6.6, and 7.6 this ratio reduces to 0.422, 0.382. 0.361, and 0 respectively. This suggests that stalk cleavage significantly affects the kinetic activities of sucrase, but that this effect is dependent on pH. Further, the ability of sucrase to function at a low pH is possibly due to the acidification of the surrounding milieu due to proton pumps within intact vesicles (Ganapathy and Leibach, 1983).

7.3.1.3 The effect of attachment to BBMV on the kinetic parameters of maltase

The stalked region of the maltolytic enzymes also appears to be important to continued maltolytic activity. Hence, from pH 4.6 - 7.6 the ratios of soluble to amphipathic maltolytic activities were ~0.6 suggesting that, following solubilisation, maintaining an intact stalk was important for continued activity. At pH 3.6 the ratio of soluble to amphipathic activity was 0.105 suggesting that maltoltyic activity was negatively affected by low pH when the stalked region was lost. Suggesting that solubilisation from the BBMV vesicle may alter the interaction of the enzymes with their substrates, but that this may not necessarily result in a change to maximal velocity.

7.3.1.4 The effect of proteolytic treatment on the kinetic parameters of ALP

Alkaline phosphatase does not have a stalked segment so is not susceptible to stalk cleavage. However, a ‘soluble’ fraction was obtained following treatment with pancreatin. Published work suggests that 13 % of ALP can be solubilised by pancreatic peptidases (Young and Das, 1990), which is in keeping with what was found here. Although the diminished activities suggest that there was also a degree of inactivation.

7.3.2 Degree of solubilisation

Bile has been shown to liberate ~ 90 % of maltase, sucrase and lactase and 45 % of ALP (Young and Das, 1990, Vasseur et al., 1978). In contrast pancreatic enzymes liberate 83,

82, 60 and 13 % of maltase, sucrase, lactase and ALP respectively (Young and Das, 1990). This is similar to the solubisation rates achieved by pancreatic elastase alone, where maltase, sucrase, lactase and ALP were solubilised 86, 72, 54, and 14 % respectively (Maestracci, 1976). The degree of peptidolytic solubilisation depends on the peptidase, *i.e.* elastase readily cleaves BB enzymes from BBMV whereas there is limited solubilisation by trypsin and chymotrypsin (Maestracci, 1976). These proportions can be used to calculate the expected activity, *i.e.* if 90 % of maltase is solubilised by bile we would expect the activity of the soluble fraction to be 90 % of BBMV fraction. Hence, the numerator would be the V_{max} obtained following bile solubilisation (*i.e.* the V_{max} of the amphipathic fraction) and the denominator would be the expected activity, *e.g.* for bile solubilised maltase it would be 90 % of the V_{max} for maltase in the BBMV fraction.

7.3.3 Actual activities *vs.* expected activities

The activities of maltase and sucrase, which are reported to have the greatest stability following bile solubilisation (Young and Das, 1990), were in keeping with previous works, *i.e.* the actual to expected activities were close to 1 (Young and Das, 1990, Vasseur et al., 1978). Maltose is hydrolysed by sucrase, isomaltase, maltase and glucoamylase (Van Beers et al., 1995a). These enzymes have different pH sensitivity which also differ depending on whether they are BBMV-bound or soluble (Auricchio et al., 1965). For example papain solubilised maltases are more tolerant of high pH, retaining up to 20 % more activity than bound counterparts (Auricchio et al., 1965)

7.3.3.1 Actual *vs.* expected sucrase activities

When the ratio of the expected to actual sucrase activities were calculated for the soluble and amphipathic fractions a clear pattern emerged (table 41) *i.e.* the ‘actual’ sucrase activities in the amphipathic fraction were 1.1 and 1.09 fold greater at pH 3.6 and 4.6 than

the estimated activities suggesting that there was a degree of activation. Likewise at pH 3.6 there was activation in the soluble fraction, *i.e.* the actual activity in the soluble fraction was 1.27 fold greater than the estimates. At pH concentrations higher than this there were fundamental losses in actual activities of pancreatin solubilised enzyme compared to estimated activities culminating in a complete loss of activity at pH 7.6.

Table 41. Ratio of actual sucrase activities to expected activities

	Actual activity/expected activity				
Fraction/pH	3.6	4.6	5.6	6.6	7.6
Bile solubilised (amphipathic)	1.099	1.087	0.592	0.810	0.465
Pancreatin solubilised (soluble)	1.268	0.521	0.256	0.332	0.000

7.3.3.2 Actual *vs*. expected maltase activity

In the amphipathic fraction the ratio of actual to expected activity was lower at pH 3.6, while all other pH concentrations activities were greater than 1 (table 42) which suggests that activation has occurred at these pH concentrations. The same pattern of activation is evident in the pancreatin solubilised enzyme suggesting that a) activation has occurred and b) that solubilised maltolytic enzymes (regardless of solubilisation method) may hydrolyse better at a higher pH.

Table 42. The ratio of actual to expected maltase activities

	Actual activity/expected activity				
pH	3.6	4.6	5.6	6.6	7.6
Bile solubilised (amphipathic)	0.634	1.153	1.068	1.028	1.057
Pancreatin solubilised (soluble)	0.074	0.789	1.184	1.139	1.172

7.3.3.3 Actual *vs.* expected lactase activities

The expected activities for amphipathic lactase at pH 5.6 and 6.6 were 143.78 and 131.04 pmol/min respectively. The ratio of these activities were 1.01 and 0.49 times those activities seen in the BBMV fraction, irrespective of pH concentration, which suggests that pH may have a role to play in whether the enzyme activity is maintained.

7.3.3.4 Actual *vs.* expected ALP activity

According to previous reports 13 % of bound ALP was expected to be solubilised by pancreatic peptidases (Young and Das, 1990), but since ALP has no stalked region to cleave, this "solubilisation" may result from shear forces, occurring during processing. There was a lower than expected ratio of actual to expected activities (table 43) following pancreatin solubilisation, and activity was eliminated at pH 10.6. The activities in the amphipathic fractions were lower than expected. Hence, expected activities were less than half of what was the expected, which suggests that there may be a degree of ALP inhibition.

Table 43. The ratio of actual to expected ALP activities

	Actual activity/expected activity		
pH	8.6	9.6	10.6
Bile solubilised	0.511	0.463	0.585
Pancreatin solubilised	0.884	0.764	0

Evidence suggests that bile solubilisation appears to be more complete than proteolytic solubilisation, which was evident in enzyme activities that were recovered in the amphipathic fraction, *i.e.* there were equivalent activities recovered in the amphipathic fraction for sucrase and maltase. Suggesting that the BBMV membranes were entirely disrupted and the enzyme liberated and recovered, which is supported by previous work that determined that 90-100 % of maltase and sucrase activity is released following bile

solubilisation, while only 82 % is recovered following proteolytic solubilisation with human pancreatic secretions (bile removed) (Young and Das, 1990). Maltase and sucrase, have been shown to be inactivated 18 and 10 % respectively following bile solubilisation (Young and Das, 1990), which is likely due to the denaturation of the maltolytic enzymes by bile (Anson, 1939). The combination of increased diffusivity may compensate for the degree of inactivation.

In contrast, when ALP was solubilised from the membrane by bile its maximal velocities were reduced 4 to 5 fold. Other work demonstrates that only 50 % of ALP is released following exposure to bile and that the solubilised enzyme was unstable, *i.e.* 56 % was inactivated (Young and Das, 1990). Hence, any solubilised enzyme may be subsequently inactivated; either by denaturation (Otzen, 2011), structural changes that result in a decrease in the rate of turnover (k_{cat}) (Fersht, 1999) or by changes in the rate of substrate/enzyme unbinding (K_{-1}) (Reuveni et al., 2014). Thus, turnover rate may be decreased by increasing the rate of substrate unbinding, leading to the initiation of a new binding (Reuveni et al., 2014). However, it has also been shown that when k_{cat} is prolonged, increasing substrate unbinding, and initiating a new catalytic attempt may increase the overall turnover rate (Reuveni et al., 2014). Such changes may be caused by structural differences in the enzyme following solubilisation that alter the binding or dissociation rate of the substrate and product respectively (Fersht, 1999). Further work would be required to elucidate the mechanism.

The maximal velocity of ALP in the soluble fraction was significantly less than the BBMV fraction. Experimental work has shown ALP is poorly solubilised, and is significantly degraded on exposure to pancreatic peptidases, with elastase degrading ALP to the greatest extent (Maestracci, 1976). Hence, it is likely that ALP is inactivated by

proteolysis rather than being solubilised due to the absence of a stalked region (The UniProt Consortium, 2015).

There were no lactase activities in the soluble fractions following exposure to pancreatin, which was in keeping with work that suggested that lactase is inherently unstable following solubilisation (Young and Das, 1990). That leads to several conclusions; firstly, loss of the anchoring region may result in a loss of lactolytic activity, but from these results it is difficult to determine whether the absence of lactase activity was due to lactase instability, as proposed. Conversely, lactase may be resistant to proteolysis, and may remain attached to BBMV if the stalked region is less than 2-3 nm (Kenny et al., 1983). No literature was found on the length of lactase's stalked region. However, research suggests that approximately 50-60 % of lactase can be released from BBMV following proteolytic hydrolysis (Young and Das, 1990, Maestracci, 1976), which suggests that it is unlikely that steric hindrance will hinder (at least some) pancreatic proteases. As duodenal secretions (bile and pancreatic secretions) or pancreatic secretions respectively obliterate, or severely diminish, the activity of lactase in bound and solubilised form (Young and Das, 1990) it may be that lactase is inherently unstable as suggested. However, in chapter 6 (figure 23), the addition of pancreatin to the BBMV fraction did not significantly alter the hydrolytic activity of lactase suggesting that lactase may still be associated with the BBMV. In retrospect this work should have assessed residual lactase activities in the pellets, arising from bile and pancreatin solubilisation, in order to assess such possibilities.

With a stalked region of 3.5 nm (Cowell et al., 1986) the proteolytic cleavage of the sucrase stalk would likely have been complete. However, at the pH concentrations of 4.6-6.6, the maximal velocities of sucrase were significantly decreased (2-5 fold) compared to the BBMV fraction. A reduction in activities infers that there was inactivation or

incomplete proteolysis, which is in contrast to other work that suggested that 82 % of sucrase is solubilised following 1 hour exposure to proteolytic treatment (Young and Das, 1990). Further, these authors determined that there is sucrase activation of 14 % following proteolytic release (Young and Das, 1990). In this work this phenomenon was only evident at pH 3.6.

The pattern of maltase release, activity and stability were similar. More maltase was recovered in the amphipathic phase than the soluble phase. This is compatible with other work, which determined that 92 % and 83 % of maltolytic enzyme is released on exposure to bile and pancreatic enzymes respectively (Young and Das, 1990). Interestingly, from pH 4.6-7.6, 100 % of maltase activity was recovered in the amphipathic phase. However, with stalked region of 2.5 nm (Norén et al., 1986) maltase (from MGAM) might not be contributing to the bulk of activity seen here. It is likely that SI, with a stalk length of 3.5 nm (Cowell et al., 1986), would be more readily hydrolysed, augmenting activity. This speculation is further supported by evidence that suggests that sucrase contributes to 80 % of maltose hydrolysis in the small intestine (Van Beers et al., 1995a).

The mechanism which anchors a BB enzyme into the BBMV membrane could influence the stability of the enzyme after solubilisation. Lactase has a type I peptide anchor, meaning it is anchored to the membrane at the *C*-terminus. Alkaline phosphatase has a GPI lipid anchor (Fosset et al., 1974). Both lactase and ALP were significantly inhibited by exposure to bile or pancreatic enzymes (Young and Das, 1990). Whether or not anchoring by the C-terminus increases an enzymes susceptibility to inactivation is not known, but there is anecdotal evidence here and elsewhere (Maestracci, 1976, Young and Das, 1990), which suggests that more investigation may be needed. It may be thought that, for ALP, possible dissociation of the dimer, following exposure to bile or pancreatin may contribute to the 6-10 fold loss in activity seen following exposure to these

treatments. However, ALP is thought to be stable in a monomeric state (Semenza, 1986). The soluble and aqueous fractions of maltase and sucrase appear to be more stable; these enzymes have type II peptide anchors.

Another consideration is the degree of glycosylation. Glycosylation may protect the tertiary structure of enzymes from enzymatic degradation (Mer et al., 1996, Kingsley et al., 1986, Vaňková et al., 1994). Lactase, ALP, sucrase and maltase are reported to be 17 (Birkenmeier and Alpers, 1974), 12 (Fosset et al., 1974), 25 (Naim et al., 1988a) and 30-40 % glycosylated respectively (Naim et al., 1988b, Kelly and Alpers, 1973). The lower glycosylation rates seen in lactase and ALP may explain why lactase and ALP are more susceptible to loss of activities.

7.3.4 The effect of pH on the activity of enzymes in different fractions

Modifying the pH levels resulted in significant changes to the K_m of the enzymes assayed. Lactase displayed the least stability in the face of changes in pH, *i.e.* there were significant decreases in lactase activities in the BBMV and amphipathic fraction with increases in pH. This is supported by published data that suggests that when the pH of papain solubilised and purified lactase varies one pH unit from the pH optimum of 6, lactase activity decreases by half (Birkenmeier and Alpers, 1974).

Alkaline phosphatase also displayed poor tolerance for changes in pH, particularly in the solublised fractions. In contrast, BBMV maltase and sucrase were relatively stable, with changes in pH, in all fractions. The most stable activity was seen in the amphipathic fraction. Hence, an intact anchoring segment may improve stability.

The pH optimum of sucrase in the BBMV fraction was 7.6. In the soluble, amphipathic and aqueous fractions the pH optimum was 3.6. The pH optimums of papain solubilised and purified rat sucrase, measured in either 50 mM sodium or the absence of sodium were

5.9 and 6.7 respectively (Kolínská and Kraml, 1972). This may suggest that the buffer used in this work did not provide sufficient sodium to establish the sodium activation that reduces the pH optimum. However, their work measured sodium activation in proteolytically solubilised and purified enzyme; so the pH optimum of 7.6 achieved here may be better representative of how sucrase behaves when attached to the BBMV membrane or is in the presence of other BBMV enzymes, *i.e.* liberation from the surface of BBMV in the presence of other enzymes may alter enzyme activity.

The liberation of BB enzymes from BBMV by bile or pancreatin may alter the dynamics of the enzymes. Hence, the increases in sucrase activities in the soluble, amphipathic and aqueous fractions at pH 3.6 (although not statistically significant) may result from structural modifications due to changes in pH (Goto et al., 1990). Although protein denaturation often can occur at an acidic pH, due to altered electrostatic interactions induced by the increase in positive charges (Anderson et al., 1990), at low pHs ionic and pH conditions may be conducive to the structural refolding of the enzyme as a result of changes in the hydrophobic interactions that influenced enzyme activity (Goto et al., 1990).

The increased activities of oligosaccharidases at pH 3.6 are interesting. These lower pH optimums may be more representative of what is happening *in vivo* as the pH in the milieu surrounding BBMV is thought to be acidified (Murer et al., 1976, Ganapathy et al., 2006). This is due to proteins, such as the sodium/hydrogen antiporter, remaining active in the BBMV membrane following vesiculation (Murer et al., 1976, Ganapathy et al., 2006). As a result the pH of the jejunal peri-apical space is lower than the luminal pH. *i.e.* pH 6.1 (Lucas, 1983) *vs.* pH 7.4 respectively (Ekmekcioglu, 2002). The pH of the microclimate, surrounding the BBMV, may be lower again as it is likely that these proteins acidify the area surrounding BBMV, where these enzymes are located.

7.4 Conclusion

In chapter five it was evident that bile and pancreatin affected BBMV-bound and aqueous enzymes in different ways. This chapter examined whether the solubilisation of BBMV enzymes by bile or pancreatin contributed to such changes. Results showed that there were significant changes in the substrate affinity and maximum velocities of enzymes following enzyme solubilisation. All of which have the potential to affect the activities of BBMV-bound enzymes in an *in vitro* environment. In most cases solubilisation was not deleterious. So regardless of subsequent changes to enzyme activities and affinities (for substrates), the activities of the requisite enzymes should be sufficient to hydrolyse the available substrate, only the rate may vary. Further, as there is continuous secretion of endogenous BB enzymes into the small intestine the addition of a second aliquot of enzyme, half way through the *in vitro* digestion process, would solve any problems arising from slowing hydrolysis. Hence, there are no significant reasons not to utilise an *ex vivo* BBMV preparation, and their associated enzymes, for use in an adjunct BB digestion phase. The next step therefore is to begin the validation process.

8 Chapter 8. Validation of the brush border phase: the digestion of plant polyphenolics compounds

Previous chapters have shown that an active, stable preparation of BB enzymes can be isolated from the mammalian small intestine via calcium precipitation and differential centrifugation, and the resulting BBMV fraction can be subjected to conditions emulating the small intestine, while retaining competent enzymatic activities. This chapter demonstrates that the adjunct BB method can be used for the *in vitro* digestion of plant polyphenolic compounds.

Polyphenolic compounds are phytochemicals that occur in a variety of fruit and vegetables (Scalbert et al., 2002). These compounds have roles in plant reproduction, growth, colour (Liu, 2004), and in protecting plants from environmental damage and predation (Scalbert et al., 2002, Liu, 2004). When consumed in the human diet polyphenolic compounds are thought behave as antioxidants (RiceEvans et al., 1996, Liu, 2004). This activity has been ascribed to their ability to act as reducing agents by donating electrons, primarily from the C-3 position hydroxyl group of the phenolic C-ring (Fraga et al., 2010, Tsao, 2010), to hydroxyl, peroxyl and peroxynitrite radicals (Heim et al., 2002). The heterocyclic structure of polyphenolics allows the charge to be delocalised (electron resonance) conferring stability to the compound whilst quenching the free radical (Heim et al., 2002). Hence, polyphenolics may be able to protect tissues from oxidative damage and secondary inflammatory changes that are implicated in the progression of a number of chronic diseases (Duarte et al., 2001), such as heart disease, diabetes, joint disease and some cancers (Wolk et al., 2001, Steppan et al., 2001, Van Gaal et al., 2006, Must et al., 1999). Polyphenolic compounds are also thought to modulate appetite. A number of polyphenolic compounds have been found to interact

with bitter taste receptors on the apical membrane of enterocytes of the small intestine (Roland et al., 2011, Jeon et al., 2008), *e.g.* T2R bitter taste receptors (Rozengurt, 2006). The bitter taste receptors involved are of the guanine nucleotide-binding regulatory protein (G-protein) coupled receptor (GPCR) super family (Rozengurt, 2006). The GPCR receptor family modulate intracellular signalling pathways that lead to cellular responses mediated by hormones and neurotransmitters (Rosenbaum et al., 2009), including vision, taste and smell (Rosenbaum et al., 2009). Different T2R receptors recognise different bitter compounds (Roland et al., 2011) and initiate signalling cascades that lead to an enteroendocrine response that induces satiety (Rozengurt, 2006). This process is thought to culminate in the up-regulation, synthesis and secretion of satiety inducing peptides such as cholecystokinin (CCK) and peptide-YY (PYY) (Jeon et al., 2008, Rozengurt, 2006). Bitter taste receptors capable of interacting with bitter polyphenolic compounds (Jang et al., 2007), are found in the stomach, pancreas and intestine (Jeon et al., 2008, Wu et al., 2002, Jang et al., 2007). Given that different bitter receptors interact with different polyphenolic compounds (Roland et al., 2011) it is crucial to understand the digestion and derivatisation of these compounds by endogenous enzymes in the small intestine and so determine the forms in which various polyphenolics would be present to interact with bitter taste receptors of the adjacent mucosa. This chapter examines the structure of polyphenolics, and what is known of their digestion and metabolism and excretion for this reason.

8.1 Structure of polyphenolic compounds

Polyphenolic compounds are characterised by the presence of one or more phenolic sub-units (Bravo, 1998, Manach et al., 2004) that can exist from monomers (*e.g.* phenolic acids) to complex polymers (*i.e.* tannins) of up to 30 kDa (Bravo, 1998). They arise from a broad group of bioactive chemicals called plant phytochemicals (Liu, 2004). Polyphenolic compounds can be classified into several classes that are all derived from the basic phenolic unit (figure 28). Of these compounds, flavonoids are the most ubiquitous, and within this group flavonols and flavones are most common (Bravo, 1998, Tsao, 2010).

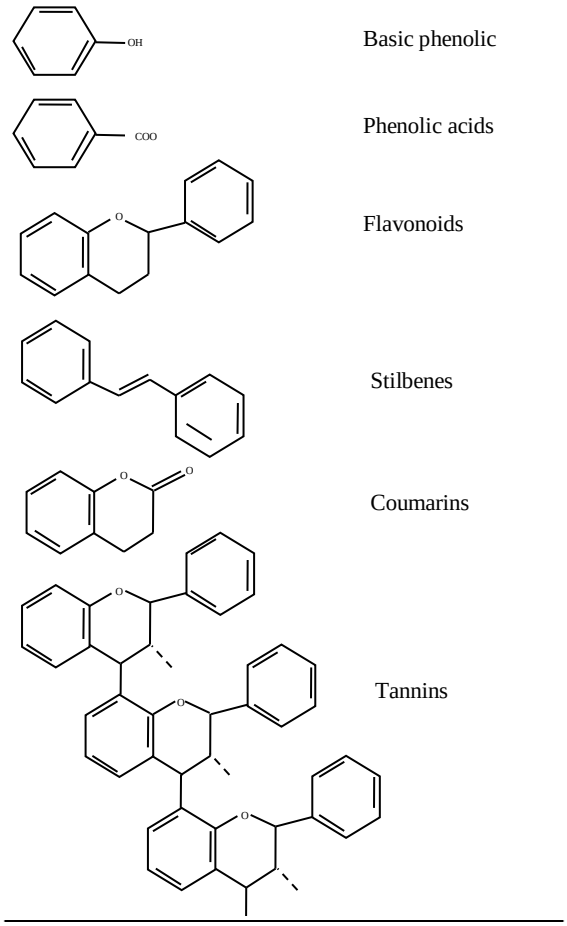

Figure 28. The most common classes of polyphenolic compounds derived from basic phenolics.

This chapter will focus primarily on the flavonol group as flavonols have been found to activate bitter taste receptors (Roland et al., 2013), and are found in commonly eaten foods, including berries, apple, grapefruit, grapes, cranberry, leek, broccoli, endive, radish, leek, onion, lettuce, tea and red wine (Rice-Evans et al., 1996). Flavonol compounds are principally conjugated with carbohydrate moieties (mono, di or oligomeric O-glycosides of primarily D-glucose, but also D-galactose, L-rhamnose, D-xylose and L-arabinose) (Herrmann, 1976, Bravo, 1998). They may also be glucuronidated, galacturonidated, methylated or sulphated

(Herrmann, 1976, Bravo, 1998) or linked to amines, lipids or other compounds (Bravo, 1998). The two stereoisomers of the various monosaccharides are linked differently to the polyphenolic compound, *i.e.* L-glycosides are added in the α-conformation and D-glycosides in the β-conformation (Herrmann, 1976). The primary bonding site for glycosidic residues in flavonols is the 3 position, but less commonly, glycosylated at the 7 position, and infrequently in the 5, 3' or 4' positions. Diglycosides are also found as 3-*O*-biosides and 3,7-di-*O*-glycosides (Herrmann, 1976).

8.2 Bioactivity and action of polyphenolic compounds as nutraceuticals and clinical agents

The conjugated structure and the hydroxylation pattern of flavonols are thought to confer antioxidant properties (Tsao, 2010) that are purported to be beneficial to health (Scalbert et al., 2005). Polyphenolic compounds are thought to scavenge free radicals, chelate metals involved in radical formation, protect membranes from lipid oxidation, modify membrane fluidity, inhibit enzymes (such as NADPH-oxidases) that produce superoxide radicals, and inhibit transcription factors involved in the inflammatory process (Fraga et al., 2010). However, it is important to understand in what form polyphenolic compound exist in nature, how they are digested by the host, and what form they are in when they are in contact with the absorptive tissue of the small intestine. It is therefore important to be able to trace their digestion in a mammalian host in order to determine whether such compounds actually possess antioxidant properties *in vivo*.

In their natural form plant polyphenolics are present predominantly as hydrophilic β-glycosides, which are not as bioactively potent (Nemeth et al., 2003b). When deglycosylated the aglycones of polyphenolic compounds display a higher degree of bioactivity. For example quercetin aglycones have greater capacity for inducing the phase

II anti-carcinogenic enzyme quinone reductase (QR) than most quercetin glycosides (Williamson et al., 1996). Quercetin aglycone and quercetin-4'-glucoside, but not quercetin-3-rutinoside, quercetin-3-glucoside or quercetin-3,4'-diglucoside induce QR (Williamson et al., 1996). Similarly, aglycones of the isoflavanoid family more readily activate the bitter taste receptors hTAS2R14 and hTAS2R39 than the glycosylated isoflavonoids, and while glycosylated isoflavones did not activate hTAS2R14, various glycosylated isoflavones activated hTAS2R39 (Roland et al., 2011).

The ability of polyphenolics to act as antioxidants *in vivo* is questionable. Following ingestion of quercetin the bioactive aglycone is not detected in plasma, and only small amounts of the bioactive polyphenolic conjugates are detected in plasma (Tsao, 2010, Manach et al., 1996). First pass metabolism by enzymes of the small intestine and liver, as well as bacterial degradation by colonic bacteria, results in minimal amounts of unmodified polyphenolics being released into systemic circulation (Donovan et al., 2001). In systemic circulation polyphenolic metabolites, such as those produced by quercetin, may bind to plasma proteins (Manach et al., 1995), which makes them unavailable for tissue utilisation as antioxidants (Boulton et al., 1998). This is supported by other research that found that when quercetin was perfused into the small intestinal lumen of live rats no free quercetin was found in systemic circulation (Crespy et al., 1999). Hence, polyphenolic compounds may have their greatest biological affect in the peri-apical space where their bioactivity may be increased following deglycosylation by LPH. The metabolites formed, subsequently tend to have less antioxidant potential than the parent polyphenolic (Olthof et al., 2003, Simons et al., 2005, Roland et al., 2011). Further work is required to clarify what derivatives are producing the beneficial antioxidant effect *in vivo.*

8.3 Degradation/digestion of polyphenolic compounds by enzymes of the small intestine

Polyphenolic glycosides are reduced to their component aglycone and glycoside to varying extents in the mouth, by endogenous or microbial enzymes, by endogenous β-glycosidase enzymes of the small intestinal BB and liver, and by bacteria in the colon (Nemeth and Piskula, 2007). Table 45 demonstrates the activities of endogenous β-glycosidase enzymes on various polyphenolic glycosides. Plant polyphenolic compounds are digested and metabolised in many sites along the digestive tract, but the main carbohydrate degrading enzymes salivary α-amylase (EC 3.2.1.1) and pancreatic α-amylase (EC 3.2.1.1), which have specificity for α-1,4 linked D-glucose residues (The UniProt Consortium, 2014) and are not capable of hydrolysing the β-linked D-glycosides or the α-linked L-glycosidic residues of plant polyphenolics. The endogenous digestion and absorption of polyphenolic compounds is mediated primarily by enzymes produced by the small intestinal enterocyte (Walle, 2004), figure 29. The luminal deglycosylation of polyphenolic glycosides occurs by the BB β-glycosidase lactase-phorizin hydrolase (LPH) (EC 3.2.1.108/3.2.1.62) (Day et al., 2000). The majority of β-glycosidase activity (75-85 %) occurs at the lactase site (EC 3.2.1.108), with the remainder of activity occurring at the phlorizin site (EC 3.2.1.62) (Day et al., 2000, Day et al., 2003). The hydrophobic aglycone, produced by deglycosylation, can then be absorbed by simple diffusion (Wiczkowski et al., 2008, Nemeth et al., 2003b). The glycoside moiety is absorbed by transcellular carrier mediated absorption or paracellular diffusion (Pappenheimer, 1993, Wright et al., 2006). In the enterocyte, cytosolic β-glycosidase (EC 3.2.1.21) is thought to deglycosylate (Berrin et al., 2003) those polyphenolic glycosides that are transported into the cell via the sodium glucose linked transporter 1 (SGLT1) protein (Wolffram et al., 2002).

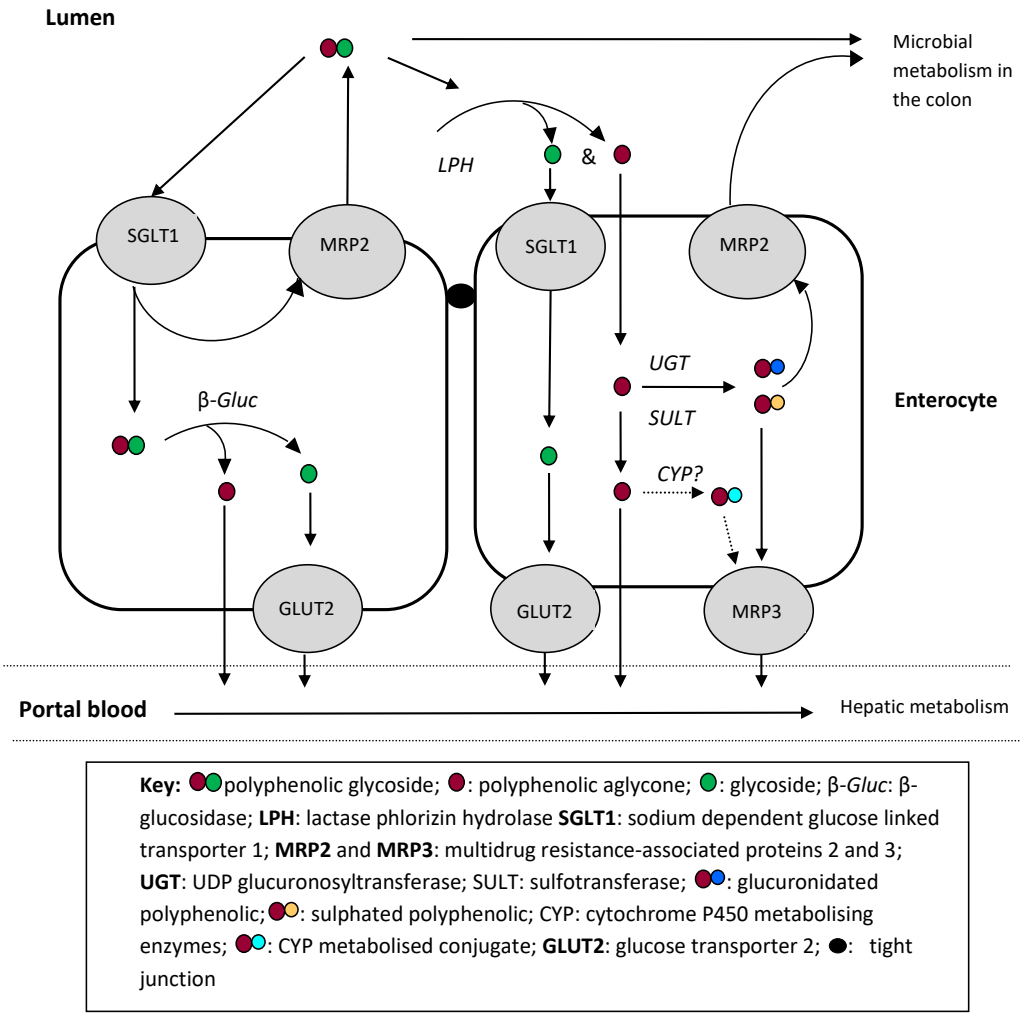

Figure 29. Schematic showing the digestion and metabolism of polyphenolic compounds in the small intestine

Table 44. Methods used for the *in vitro* digestion of polyphenolic compounds

Sample	Measurement parameter	Digestive phases	Enzymes/chemicals	Digestion times	Author
Red grapes	Flavonoid content	Buccal	-	1min (blender)	(Tagliazucchi et al., 2010)
		Gastric	NaCl	2hours	
			Pepsin (300 U/ml)		
			HCl		
		Pancreatic	$NaHCO_3$	2hours	
			Pancreatin (0.8g/L)		
			Bile salts (25mg/ml)		
			CaCo-2 cells		
Grape seed	Phenolic compounds	Buccal	Human salivary	10 minutes	(Laurent et al., 2007)
		Cytoplasmic enzymes	α-amylase (1,000-1,500U/mg protein)		
		Gastric	HCl	1 hour	
			Pepsin (25mg/ml)		
		Pancreatic	$NaHCO_3$	1.5 hours	
			Pancreatin (2g/L)		
			Bile extract (12g/L)		
		Intestinal	CaCo-2 cells	1.5 hours (with pancreatic)	
Broccoli	Flavonoids	Gastric	HCl	2 hours	(Vallejo et al., 2004)
	Glucosinolates		Pepsin		
	Vitamin C	Pancreatic	$NaHCO_3$	2.5 hours	
			Pancreatin		
			Bile salts		
Sweet cherries	Phenolics	Gastric	HCl	2 hours	(Fazzari et al., 2008)
	Anthocyanins		Pepsin (31500 units)		
		Pancreatic	$NaHCO_3$	-	
			Pancreatin (4mg/ml)		
			Bile acids (25mg/ml)		

Sample	Measurement parameter	Digestive phases	Enzymes/chemicals	Digestion times	References
Orange juice	Flavones	Gastric	HCl Pepsin (15,750 U)	2 hours	(Gil-Izquierdo et al., 2003)
		Pancreatic	$NaHCO_3$ Pancreatin/ bile extract (5 ml)	2 hours	
Grape Chokeberry	Polyphenols	Gastric	HCl Pepsin (60,000 U)	4 hours	(Gumienna et al., 2011)
		Pancreatic	$NaHCO_3$ Pancreatin (0.02g/10ml) Bile salts (0.12g/10ml)	2 hours	
		Large intestine	Faecal flora $NaHCO_3$		
Carob flour	Soluble fibre	Mouth	α-amylase (100-300ud/g)	5 minutes	(Ortega et al., 2011)
	Soluble sugars Polyphenols	Gastric	HCl (0.1N) Pepsin (24750ud/g)	2 hours	
		Pancreatic	$NaHCO_3$ Pancreatin (8 g/L) Bile salts (2.5ml)	2 hours	
Almond skin	Lipids Protein Polyphenols	Gastric	HCl Pepsin (146 U/ml) Phosphatidyl choline vesicle suspension (2.4mmol/L) Gastric lipase analogue(60 U/ml)	2 hours	(Mandalari et al., 2010)
		Pancreatic	$CaCl_2$ (11.7mmol//L) Sodium taurocholate (4mmol/L) Sodium glycodeoxycholate (4mmol/L) α-chymotrypsin (5.9 U/ml) Colipase (3.2µg/ml) Pancreatic lipase (54U/ml) α-amylase (25U/ml)	1 hour	
Berry fruit	Polyphenols	Gastric	HCl Pepsin (315 U/ml)	2 hours	(McDougall et al., 2007)
		Pancreatic	NaHCO3 Pancreatin (4mg/ml) Bile salts (25mg/ml)	2 hours	

Sample	Measurement parameter	Digestive phases	Enzymes/chemicals	Digestion times	References
Green tea, iron with meat, ascorbic acid or casein	Antioxidant capacity of polyphenols	Gastric	HCl Pepsin (1ml)	2 hours	
		Pancreatic	Pancreatin (0.2 g/100ml) Bile extract (1.2 g/100ml) NaHCO3	2 hours	
Bran enriched bread	Phenolic acids	Buccal	Gastric electrolytes α-amylase (5.5 mg/150ml)	.	(Hemery et al., 2010)
		Gastric (TNO)	HCl Pepsin lipase gastric electrolytes	$T_{1/2}$ 70	
		Pancreatic	NaHCO3	6 hours (aliquots hourly)	
Green tea	Recovery of catechins	Gastric (N_2)	HCl Pepsin (40 mg/ml)	1 hour	
		Pancreatic (N_2)	$NaHCO_3$ Lipase (1 mg/ml) Pancreatin (2 mg/ml) Bile (12 mg/ml)	2 hours	
Vegetable juice	Antioxidant activity Polyphenols	Gastric	HCl Pepsin (0.04mg/ml)	1 hour	(Wootton-Beard et al., 2011)
		Pancreatic	NaHCO3 Glycodeoxycholate (0.04 g/ml) Taurodeoxycholate (0.025 g/ml) Taurocholate (0.04 g/ml) Pancreatin 0.04g/500ul)	2 hours	
Cocoa	Bioavailability of epicatechins	Buccal	NaCl (0.15M)	-	(Neilson et al., 2009)
		Gastric (N_2)	HCl Pepsin (40 mg/ml)	1 hour	
		Pancreatic (N_2)	NaOH Bile solution (48 mg/ml) Pancreatin/lipase (400 mg/ml)	2 hours	

Sample	Measurement parameter	Digestive phases	Enzymes/chemicals	Digestion times	References
Apple	Metabolism of polyphenols	Buccal	Human saliva (unstim)	5 minutes	(Kahle et al., 2011)
		Gastric (N_2)	NaCl (70mM) KCl (50mM) Pepsin (0.14mM) HCl	0.3 – 4 hours	
		Pancreatic (N_2)	$NaHCO_3$ Pancreatin (1g/0.5L) Bile extract (6g/0.5L) NaCl (60mM) KCl (2.3mM)	0 - 24 hours	
		Duodenal (N_2/CO_2)	Ileostomy fluid	0 - 24 hours (dark)	

The glycosides formed are transferred into systemic circulation by the GLUT2 transporter while the aglycone may either passively diffuse through the basolateral membrane into systemic circulation or more likely is detoxified and conjugated in the cell and exported into the intestinal lumen or systemic circulation (Walle, 2004).

A study following the digestion of quercetin glycosides in ileostomy patients determined that 65-81 % of ingested quercetin glycosides were absorbed in the small intestine (Walle et al., 2000). Analysis of ileostomy effluent established that quercetin 4' glucoside and quercetin 3,4'-glucoside were completely deglycosylated in the small intestine (Walle et al., 2000). Only aglycone and small amounts of glucuronidated quercetin were recovered in the ileostomy effluent (Walle et al., 2000); the glucuronidation occurring in the enterocyte (Wong et al., 2010) and the product is subsequently effluxed into luminal contents (Mottino et al., 2000). Another study followed the *in vivo* degradation of C-4 ^{14}C labelled quercetin; examination of urine found that 3.3-5.7 % of the labelled carbon was disposed of in the urine, 0.2-4.6 % in faeces while 23-81 % was recovered in expired air (Walle et al., 2001). The labelled ^{14}C appeared in expired air as CO_2 from 4 hours, and up to 8 hours after ingestion (Walle et al., 2001). Interpretation of these results involves understanding the physiology of detoxification. Smart pill analysis of human post-prandial gastrointestinal transit time of a solid meal determined that mean gastric emptying time was 3.1 ± 0.9 hours, small intestinal residence was 5.5 ± 0.65 hours and colonic residence was 27.6 ± 15.6 hours (Maqbool et al., 2009). Other smart pill data found that mean post-prandial gastric emptying time for a solid meal was 3 hours (range 2.5-3.9), small intestinal residence was 3.8 hours (range 3.2-4.7) and colonic residence time was 21.7 hours (range 15.5-37.3) (Rao et al., 2009). Hence, gastric and small intestinal residence is likely to be approximately 7- 9 hours (Rao et al., 2009, Maqbool et al., 2009) suggesting that the ^{14}C enriched air at 4-8 hrs was evidence of primarily small intestinal metabolism and absorption, possibly with some degree of hepatic detoxification. Alternately, conjugated polyphenolics are transported into the circulatory system by multi resistance-associated protein 3 (MRP3) for further processing by hepatic

enzymes, and are subsequently excreted in urine or bile (Scalbert and Williamson, 2000, Walle, 2004).

8.3.1 Detoxification of polyphenolic compounds in the small intestine

The small intestinal detoxification of xenobiotics is a three step process involving the deglycosylation, biochemical modification, conjugation and elimination of potentially harmful compounds. This process limits the absorption and systemic circulation of potentially harmful compounds (Kaminsky and Fasco, 1992).

Phase I metabolism

The first step in the detoxification of ingested xenobiotics is termed phase I metabolism. This process involves the chemical modification of predominantly hydrophobic xenobiotics by oxidative methods that include hydroxylation, epoxidation, and dealkylation (Meunier et al., 2004). Enzymes involved in this process include alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidase, amine oxidase, aromatases, and enzymes of the cytochrome P450 family (Doherty and Charman, 2002). This process can decrease (Conney, 2003) or increase the toxicity of the metabolite (Ding and Kaminsky, 2003, Conney, 2003). The prominent phase I enzymes are the cytochrome p450 enzymes (Kaminsky and Zhang, 2003) of which cytochrome P450 3A4 (CYP3A4) is the most prevalent (Kaminsky and Zhang, 2003, Paine et al., 1997). Cytochrome P450 3A4 is an enzyme found primarily in the endoplasmic reticulum of the proximal small intestine (Paine et al., 1997, Kaminsky and Fasco, 1992). Once xenobiotics are biochemically modified the metabolites are more readily conjugated by phase II metabolising enzymes.

Phase II metabolism

In the enterocyte, the majority of polyphenolic aglycones arising from absorption or cytosolic metabolism undergo uronidation, sulfation or glutathione conjugation by cytosolic phase II enzymes such as UDP-glucuronosyltransferase, sulfotransferase (Walle, 2004, Scalbert and Williamson, 2000) and glutathione-*S*-transferase respectively (Scheepens et al., 2010). Other polyphenolics, such as the flavanol catechin, undergo cytosolic methylation (Donovan et al., 2001). The conjugated polyphenolic compounds are then exported by multi resistance-associated protein 2 (MRP2) into luminal contents for further processing by colonic bacteria and/or excretion (Scalbert and Williamson, 2000, Walle, 2004). In addition to compounds conjugated to glucuronic acid, glutathione and sulfate (Mottino et al., 2000) MRP2 can also transport non-anionic polyphenolic glycosides, transported into the cell by SGLT1, back into the intestinal lumen (Walle and Walle, 2003, Walgren et al., 2000), where they are further degraded by resident microbiota in the large intestine (Olthof et al., 2003, Simons et al., 2005). The biotransformed xenobiotics can also be transported to the liver for further processing and excretion (Walle, 2004, Day et al., 1998, Kahle et al., 2011).

8.4 Are current *in vitro* digestion models appropriate for quantifying the polyphenolic content and bioavailability of dietary polyphenolics?

The digestion and metabolism of plant polyphenols, and the appearance of bioactive derivatives, is often studied using *in vitro* digestion, a number of such studies are listed in table 44, and it is important to note that these studies did not use BB enzymes. The absence of a BB phase of digestion, particularly the lack of a β-glycosidase will result in experimental data that may not be a good representation of what occurs *in vivo*.

The rate at which polyphenolic compounds are absorbed and metabolised post-absorption will be dependent on the biochemical form in which a polyphenolic compound reaches the small intestinal mucosa. This may affect bioavailability. As mentioned earlier LPH deglycosylates polyphenolic compounds, which results in a lipophilic aglycone that passively diffuses into enterocytes (Nemeth et al., 2003b); while other polyphenolics may be transported into the cell via SLGT1 (Wolffram et al., 2002). All *in vitro* systems are unable to account for post-absorptional modifications, such as Phase I metabolism in the enterocyte (Doherty and Charman, 2002). Hence, no post translationally modified (*e.g.* sulfated or glucuronidated metabolites) will be effluxed back into luminal contents and be evident in the final digestion.

The use of ileostomy effluent from patients who have had their large intestine removed is another method used in the study of polyphenolic digestion and bioavailability. Again absorption and cytosolic and/or hepatic modification and excretion complicates analysis, but in regards to colonic metabolism this gives us a clearer picture of what polyphenolics are available to the large intestine.

In summary, current methods of *in vitro* digestion do not adequately replicate the digestion of polyphenolic compounds *in vivo*. The first step to increasing the validity of the *in vitro* digestion of polyphenolic compounds will be the incorporation of a BB phase of digestion as an adjunct to current methodology. However, accounting for post-absorptional modifications of polyphenolic compounds may be a difficult task to complete.

8.5 The digestion of polyphenolic compounds using the adjunct BB digestion phase of digestion

Investigating the structure, digestion, metabolism and disposal of plant polyphenolic compounds in the mammalian small intestine are of interest to researchers investigating dietary components

of polyphenolics that modulate appetite control. Such compounds are thought to interact with bitter taste receptors in the small intestine, and initiate signalling cascades that induce an enteroendocrine response that triggers satiety. It was important to understand the extent of endogenous and exogenous polyphenolic digestion to determine whether it is the parent polyphenolic or its metabolites that have bioactivity. This chapter assesses the validity of the adjunct *in vitro* BB digestion system, using polyphenolic compounds as the model substrates.

Table 45. Endogenous digestion of plant polyphenolics by intestinal lactase-phlorizin hydrolase, cytosolic enterocytic β-Glucosidase and hepatic β-Glucosidase.

Tissue	Species	Substrate	Enzyme	Specific activity (µmol/ min/mg)	Author
BBMV †	Ovine	Apigenin-7-glucoside	LPH‡	4.18	(Nemeth et al., 2003b)
BBMV	Human	Apigenin-7-glucoside	LPH	1.35	(Day et al., 1998)
Cytosolic §	Human	Apigenin-7-glucoside	β-Gluc¥	1.3	(Berrin et al., 2003)
Liver	Porcine	Apigenin-7-glucoside	β-Gluc	* 0.52	(Lambert et al., 1999)
Liver	Human	Apigenin-7-glucoside	β-Gluc	0.61	(Day et al., 1998)
BBMV	Ovine	Cyanidin-3-galactoside	LPH	0	(Nemeth et al., 2003b)
BBMV	Ovine	Cyanidin-3-glucoside	LPH	0	(Nemeth et al., 2003b)
BBMV	Ovine	Daidzein-7-glucoside	LPH	0.9	(Day et al., 2000)
BBMV	Ovine	Daidzein-7-glucoside	LPH	0.46	(Nemeth et al., 2003b)
BBMV	Human	Daidzein-7-glucoside	LPH	2.4	(Day et al., 1998)
Cytosolic	Human	Daidzein-7-glucoside	β-Gluc	0.76	(Berrin et al., 2003)
Liver	Porcine	Daidzein-7-glucoside	β-Gluc	*1.1	(Lambert et al., 1999)
Liver	Human	Daidzein-7-glucoside	β-Gluc	0.54	(Day et al., 1998)
BBMV	Ovine	Eridictyol-7-glucoside	LPH	3.21	(Nemeth et al., 2003b)
Cytosolic	Human	Eriodictyol-7-glucoside	β-Gluc	0.9	(Berrin et al., 2003)
BBMV	Ovine	Genistein-7-glucoside	LPH	1.99	(Nemeth et al., 2003b)
BBMV	Ovine	Genistein-7-glucoside	LPH	2.8	(Day et al., 2000)
BBMV	Human	Genistein-7-glucoside	LPH	3.15	(Day et al., 1998)
Liver	Porcine	Genistein-7-glucoside	β-Gluc	*0.91	(Lambert et al., 1999)
Liver	Human	Genistein-7-glucoside	β-Gluc	1.06	(Day et al., 1998)
BBMV	Ovine	Kaempferol-3-glucoside	LPH	3.65	(Nemeth et al., 2003b)
BBMV	Human	Kaempferol-3-glucoside	LPH	0	(Day et al., 1998)
Liver	Porcine	Kaempferol-3-glucoside	β-Gluc	0	(Lambert et al., 1999)
Liver	Human	Kaempferol-3-glucoside	β-Gluc	0	(Day et al., 1998)
BBMV	Ovine	Kaempferol-3-glucuronic acid	LPH	0.22	(Nemeth et al., 2003b)
BBMV	Ovine	Kaempferol-3-robinoside-7-rhamnoside	LPH	0	(Nemeth et al., 2003b)
BBMV	Ovine	Luteolin-4'7-diglucoside	LPH	0.64	(Nemeth et al., 2003b)
BBMV	Ovine	Luteolin-4'-glucoside	LPH	2.23	(Nemeth et al., 2003b)
Cytosolic	Human	Luteolin-4'-glucoside	β-Gluc	1.3	(Berrin et al., 2003)
BBMV	Ovine	Luteolin-7-glucoside	LPH	2.52	(Nemeth et al., 2003b)
Cytosolic	Human	Luteolin-7-glucoside	β-Gluc	2.85	(Berrin et al., 2003)
BBMV	Ovine	Naringenin-7-glucoside	LPH	3.64	(Nemeth et al., 2003b)

Tissue	Species	Substrate	Enzyme	Specific activity (µmol/ min/mg)	Author
BBMV	Human	Naringenin-7-glucoside	LPH	1.05	(Day et al., 1998)
Cytosolic	Human	Naringenin-7-glucoside	β-Gluc	0.93	(Berrin et al., 2003)
Liver	Porcine	Naringenin-7-glucoside	β-Gluc	*0.44	(Lambert et al., 1999)
Liver	Human	Naringenin-7-glucoside	β-Gluc	0.3	(Day et al., 1998)
BBMV	Ovine	Naringenin-7-neohesperidoside	LPH	0	(Nemeth et al., 2003b)
BBMV	Human	Naringenin-7-rhamnoglucoside	LPH	0	(Day et al., 1998)
Liver	Porcine	Naringenin-7-rhamnoglucoside	β-Gluc	0	(Lambert et al., 1999)
Liver	Human	Naringenin-7-rhamnoglucoside	β-Gluc	0	(Day et al., 1998)
BBMV	Ovine	Oleuropein	LPH	0	(Nemeth et al., 2003b)
BBMV	Ovine	Q-3,4' - diglucoside	LPH	0.16	(Nemeth et al., 2003b)
BBMV	Ovine	Q-3,4'-diglucoside	LPH	0.28	(Nemeth et al., 2003b)
BBMV	Human	Q-3,4'-diglucoside	LPH	0	(Day et al., 1998)
Liver	Porcine	Q-3,4'-diglucoside	β-Gluc	0	(Lambert et al., 1999)
Liver	Human	Q-3,4'-diglucoside	β-Gluc	0	(Day et al., 1998)
BBMV	Rat	Q-3-arabinoside	LPH	0	(Arts et al., 2004)
BBMV	Ovine	Q-3-arabinoside	LPH	0	(Nemeth et al., 2003b)
BBMV	Rat	Q-3-galactoside	LPH	0	Arts 2004
BBMV	Ovine	Q-3-galactoside	LPH	0.13	(Nemeth et al., 2003b)
BBMV	Rat	Q-3-glucoside	LPH	Hydrolysed	(Arts et al., 2004)
BBMV	Ovine	Q-3-glucoside	LPH	3.16	(Nemeth et al., 2003b)
BBMV	Ovine	Q-3-glucoside	LPH	2.7	Day et al 2000
BBMV	Human	Q-3-glucoside	LPH	0.14	(Day et al., 1998)
Liver	Porcine	Q-3-glucoside	β-Gluc	0	(Lambert et al., 1999)
Liver	Human	Q-3-glucoside	β-Gluc	0	(Day et al., 1998)
BBMV	Ovine	Q-3-malonylglucoside	LPH	0.22	(Nemeth et al., 2003b)
BBMV	Rat	Q-3-rhamnoglucoside	LPH	Jejunum: 10.22	(Ioku et al., 1998)#
BBMV	Ovine	Q-3-rhamnoglucoside	LPH	0	(Nemeth et al., 2003b)
BBMV	Human	Q-3-rhamnoglucoside	LPH	0	(Day et al., 1998)
Liver	Human	Q-3-rhamnoglucoside	β-Gluc	0	(Day et al., 1998)
BBMV	Rat	Q-3-rhamnoside	LPH	0	(Arts et al., 2004)
Liver	Porcine	Q-3-rhamoside	β-Gluc	0	(Lambert et al., 1999)
BBMV	Ovine	Q-3-xyloside	LPH	0	(Nemeth et al., 2003b)
BBMV	Rat	Q-4'-glucoside	LPH	Hydrolysed	(Arts et al., 2004)

Tissue	Species	Substrate	Enzyme	Specific activity (µmol/ min/mg)	Author
BBMV	Rat	Q-4'-glucoside	LPH	Jejunum: 59.17	(Ioku et al., 1998)#
BBMV	Ovine	Q-4'-glucoside	LPH	3.08	(Nemeth et al., 2003b)
BBMV	Ovine	Q-4'-glucoside	LPH	3.2	(Day et al., 2000)
BBMV	Human	Q-4'-glucoside	LPH	2.14	(Day et al., 1998)
Cytosolic	Human	Q-4'-glucoside	β-Gluc	1.19	(Berrin et al., 2003)
Liver	Human	Q-4'-glucoside	β-Gluc	0.74	(Day et al., 1998)
Liver	Porcine	Q-4'-glucoside	β-Gluc	*1.51	(Lambert et al., 1999)
BBMV	Rat	Q-7-glucoside	LPH	Jejunum: 3.52	(Ioku et al., 1998)#
Cytosolic	Human	Q-7-glucoside	β-Gluc	0.77	(Berrin et al., 2003)
BBMV	Ovine	Q-3-rhamnoside	LPH	0	(Nemeth et al., 2003b)

#: This method is likely to yield a mixture of β-gluc and LPH, which may distort results; *Vmax in µmol/min/mg; †BBMV: brush border membrane vesicle; §: Cytosolic: cytosolic enzyme of the small intestinal enterocyte; ‡LPH: lactase-phlorizin hydrolase (EC3.2.1.108/ 3.2.1.62); ¥: β-gluc: β-glucosidase (EC 3.2.1.21)

8.6 Methods

8.6.1 Reagents

Quercetin-3-Rutinoside (1139S) quercetin-3-glucoside (1327S) were obtained from Extrasynthese. An ethanol/water extract of onion flesh polyphenolics (AC110386-2) was kindly donated by Patricia Davis of Plant and Food Research, Lincoln, New Zealand. Other reagents are detailed in section 3.1.

8.6.2 Preparation of the BBMV fraction

Small intestinal mucosal scrapings were obtained from eight 10 week old male Sprague Dawley rats. The husbandry and procedures undertaken (AEC 12687) are described in section 3.2. The intestinal scrapings of these rats were used to prepare the BBMV and aqueous fraction using a modified version of the calcium precipitation and differential centrifugation method (Boutrou et al., 2008, Kessler et al., 1978, Sakuma et al., 2009) (figure 4), at a relative concentration of 33.33 mg/ml.

8.6.3 Preparation of the pancreatic fraction

The pancreatic fraction was prepared using the concentration and rationale described in section 4.3.1, and kept on ice until required.

8.6.4 Preparation of the biliary fraction

The biliary fraction prepared using the concentration and rationale described in section 4.3.2.

8.6.5 Preparation of the polyphenolic compounds

8.6.5.1 Internal standard

The internal standard epicatechin was prepared in methanol at a concentration of 5 µg/ml, and was added 1:1 to samples following in *in vitro* digestion.

8.6.5.2 Purified polyphenolic compounds

Quercetin-3-glucoside (Q3G) and quercetin-3-rutinoside (Q4G) were dissolved in methanol, and were present in reaction vessels at a concentration of 4.05 µg/ml.

8.6.5.3 Onion flesh extract

The onion flesh extract contained the following flavonoids quercetin-3,4′-diglucoside (Q34DI), quercetin-4′-glucoside (Q4G), isorhamnetin, isorhamnetin-4′-glucoside (I4G), quercetin-3-glucoside (Q3G) and quercetin. Preliminary characterisation of the onion flesh extract showed that Q-4'-glucoside (Q4G) was present at the highest concentration (53 µg/per mg of extract). The onion flesh polyphenolic extract was dissolved in methanol to a final concentration of 6.79 mg/ml, equating to 0.36 µg/µl Q4G (final concentration of 10.6 µg/ml in the reaction vessel), which was within the detection limits of the LC-MS (1-20 µg/ml). The onion flesh polyphenolic extract was diluted based on the concentration of the polyphenolic with the highest concentration.

8.6.6 Brush border *in vitro* digestion of plant polyphenolics

The first step to assess whether the *in vitro* BB digestion model was valid for use in the digestion of digestive components and whole foods was to determine the fate of polyphenolic glycosides during BB *in vitro* digestion. This was achieved in two ways. Firstly, polyphenolic glycosides present in onion extract were exposed to BB preparations alone and their digestion tracked over 2 hours. Secondly, the fate of a positive (quercetin-3-glucoside) and a negative control (quercetin-3-rutinoside) were assessed under the conditions of small intestinal *in vitro* digestion, *i.e.* in the presence of physiological concentrations of pancreatic, biliary and BB secretions. These conditions were used as it was important to see whether polyphenolic compounds known to be deglycosylated by BB LPH (*i.e.* Q3G) were digested, and to compare that with a polyphenolic compound that is known to not to be deglycosylated by BB LPH (*i.e.* Q3R). It was necessary to see

whether pancreatin with or in the absence of bile affected the activity of BB LPH, and to determine whether pancreatin or bile themselves induced deglycosylation of the quercetin glycosides or effected the structural integrity Q3G and Q3R. Controls also included heat treated BBMV (HBBMV) with and without biliopancreatic secretions. Purified quercetin-3-glucoside and quercetin-3-rutinoside were added to BBMV and HBBMV as positive and negative controls for the digestion of onion polyphenolic compounds (small intestinal conditions). All reactions were undertaken in triplicate.

Temporal changes in the concentration of onion flesh polyphenolics were calculated relative to time zero.

The digestion of Q3G and Q3R in the presence of bile and pancreatin were calculated in µg/ml

One hundred microliters of epicatechin (5 µg/ml methanol) was added to 100 µl aliquots of digestate at each sampling time and these samples were immediately frozen at – 20 °C until ready for analysis.

8.6.6.1 Brush border *in vitro* digestion

Aliquots of buffer, and onion polyphenolics, or purified polyphenolics plus methanol were added to a 2 ml capacity 96 well plate, all kept below 4ºC by using a cold (-20 °C) Nunc retention plate (ThermoFisher, Auckland, NZ). Aliquots of pancreatin and bile were added, followed by BBMV or HBBMV preparation. The heat treated samples contained BBMV fraction that had been heated at 95 ºC for 30 minutes, and then cooled on ice. The volumes of these components are shown in table 46. Aliquots of 100 µl were taken at time zero and added to 100 µl of methanol containing the internal standard epicatechin (5 µg/ml).

Following removal of the time zero aliquots the reaction vessel was incubated at 37ºC on an orbital shaker (80 rpm) for 2 hours, 100 µl samples were taken every 30 minutes and frozen immediately at -20 ºC until analysis. At 60 minutes 261 µl of BBMV (pH 8) was added and the pH of the incubation was raised to pH 8 with total volume change of 267 µl. This BBMV was added in buffer of pH 8 to mimic pH differences in the peri-apical space and the pH requirements of the peptidase enzymes. The pH of samples were aseptically checked, and the pH adjusted to pH 8 (6 µl of Na_2HPO_4). The change of dilution was accounted for during the analysis of results.

Table 46. Composition of digestive secretions used for *in vitro* digestions

Digestion	**Onion polyphenolic**	**Purified polyphenolics**
Secretion	**Volume (µl)**	**Volume (µl)**
Buffer	1.195	822
Pancreatic secretions	0	243
Bile	0	130.3
BBMV secretion	261	261
Methanol	0	32.02
Polyphenolics	44	12.16*
Total volume	1500	1500

*6.08 µl of quercetin-3-glucoside and 6.08 µl of quercetin-3-rutinoside

8.6.7 LC-MS method

See section 3.6 for details.

8.6.8 Statistical analysis

Data are from a time-course experiment (0 to 120 minutes). Samples are mixed with either brush-border membrane (BBMV) or heat-treated (deactivated) BBMV (HBBMV) and either nothing else, bile, pancreatin, or bile and pancreatin. The levels of several compounds were measured using LC-MS. There were three replicates of each treatment combination, which were repeatedly sampled. The data was analysed using Genstat using a repeated measures ANOVA (split-plot design with Greenhouse-Geisser epsilon

estimated and used as a multiplier for the 'repeated' effects), and post-hoc Bonferroni adjusted Fisher least significant differences were used to determine significant differences between treatment. The I-bars for between treatment comparisons (large I-bar) and between times within treatments comparison (small I-bar) are set at p = 0.0005 (approximately p = 0.05, Bonferroni adjusted) For the metabolite quercetin the I-bars are set at p = 0.001 (approximately p = 0.05, Bonferroni adjusted).

8.7 Results

8.7.1 Digestion of onion flesh polyphenolic compounds by the BB digestive phase in the absence of bile and pancreatin

Both the BBMV and the HBBMV reaction mixtures developed a yellow tint during the course of the *in vitro* digestion. This was more pronounced in the BBMV sample, and after the pH change at 1 hour.

The relative concentrations of Q34DI, Q4G, Q3G, I4G and quercetin and isorhamnetin and the internal standard are shown in figure 30; and the relative concentrations of the positive control (Q3G), negative control (Q3R) and their internal standard (epicatechin) are shown in figure 31.

8.7.1.1 Epicatechin (internal standard) (onion extract)

There were no significant differences in the relative concentrations of epicatechin between the BBMV and the HBBMV fractions and relative concentrations did not change significantly over time.

8.7.1.2 Quercetin-3-glucoside (onion extract)

There were no significant differences in the relative concentration of Q3G between the BBMV and HBBMV fractions or over time. Even though the relative concentrations of

Q3G in the BBMV, and to a lesser extent the HBBMV fraction appeared to increase over time due to high variability in samples.

8.7.1.3 Quercetin (onion extract)

In the absence of active BB enzyme, quercetin levels were below the detection limit in the HBBMV fraction, and there was insufficient data to calculate relative concentration. There were no significant differences in the relative concentrations of quercetin in the BBMV fraction.

8.7.1.4 Quercetin-3-rutinoside (onion extract)

No Q3R was found in the BBMV or HBBMV fractions even though onion flesh is reported contain Q3R in the range 0.18-1.36 mg/100g (Neveu et al., 2010). None was found in our sample.

8.7.1.5 Isorhamnetin-4′-glucoside (onion extract)

There were significant differences in the relative concentration of isorhamnetin-4′-glucoside in the BBMV and HBBMV fractions (d.f. 1,23, $F=91.41$, $p<0.001$). The relative concentration of isorhamnetin-4′-glucoside in the BBMV fraction significantly decreased over time (d.f. 3,23, $F=19.33$, $p<0.001$) while it did not in the HBBMV fraction. Hence, there was a significant time x fraction effect in the BBMV fraction (d.f. 3,23, $F=22.72$, $p=0.002$).

8.7.1.6 Isorhamnetin (onion extract)

There were no significant differences in the concentration of isorhamnetin across time in the BBMV fraction. Isorhamnetin is formed following the deglycosylation of isorhamnetin-4′-glucoside into isorhamnetin and glucose, so is present in measurable concentrations in the BBMV fraction. The concentration of isorhamnetin was very low in

the HBBMV fraction suggesting that isorhamnetin was present in very low concentrations in the onion extract, and therefore not able to be measured.

8.7.1.7 Quercetin-3,4′-diglucoside (onion extract)

It appears as though Q34DI is not hydrolysed by LPH. There appeared to be a significant increase in the relative concentration of 3,4′-diglucoside between 60 and 90 minutes in the BBMV fraction (d.f. 3,23, $F=9.65$, $p=0.014$), driving an apparent significant difference between the fractions (d.f. 1,23, $F=18.73$, $p=0.012$). However, post-hoc Bonferroni comparisons do not suggest there are any differences in relative concentration at each time point.

8.7.1.8 Quercetin-4′-glucoside (onion extract)

There were large significant differences in the relative concentration of Q4G between the BBMV and HBBMV fractions (d.f. 1,23, $F=483.17$ $p<0.001$). The relative concentration of Q4G in the HBBMV fraction remained the same throughout the assay, as expected. There was however as significant time x fraction effect in the BBMV fraction (d.f. 3,23, $F=6.14$, $p=0.038$). The relative concentration of Q4G dropped 4.15 fold from time zero to 30 minutes, and decreased a further 15 fold by 2 hours.

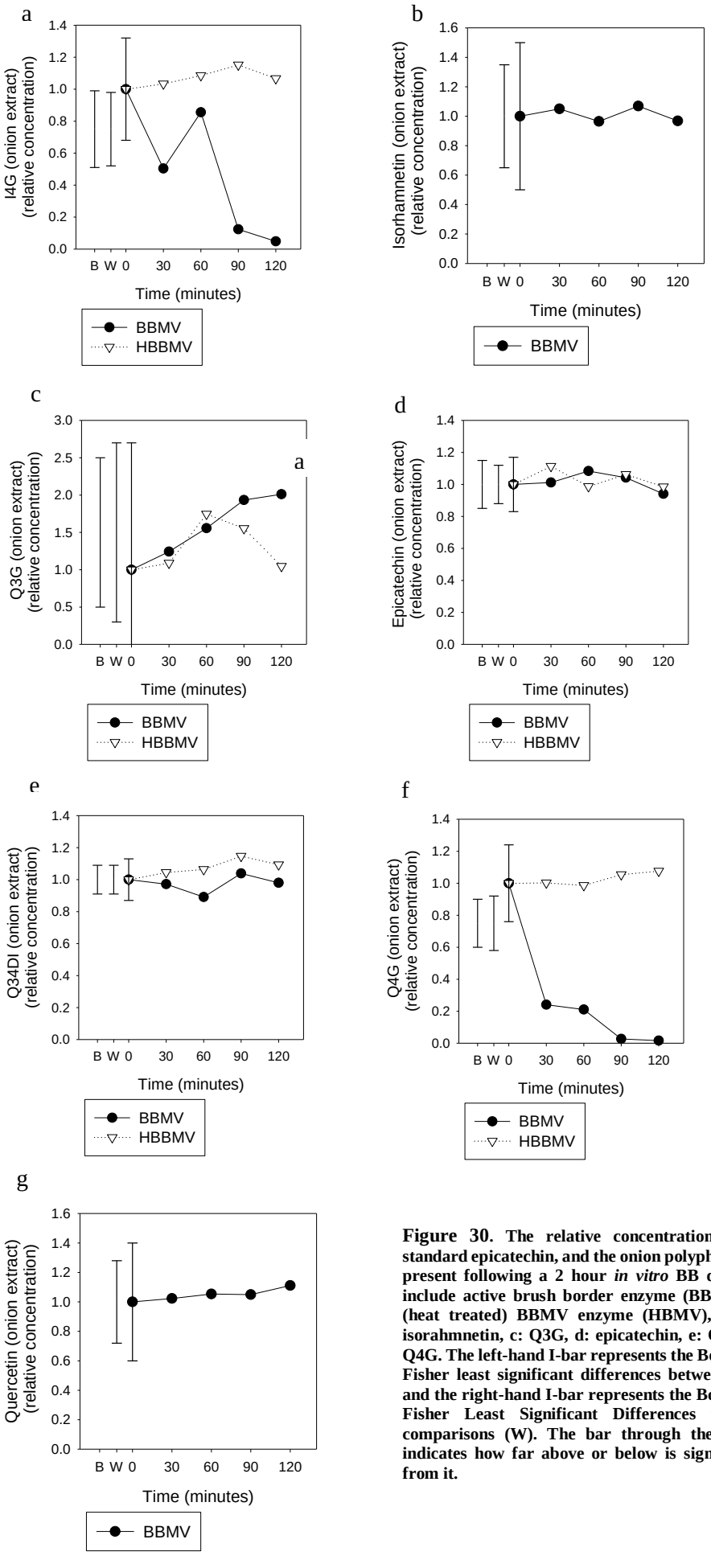

Figure 30. The relative concentration of the internal standard epicatechin, and the onion polyphenolic compounds present following a 2 hour *in vitro* BB digestion. Samples include active brush border enzyme (BBMV) and inactive (heat treated) BBMV enzyme (HBMV), n=3. a: I4G, b: isorahmnetin, c: Q3G, d: epicatechin, e: Q34DI, f: Q4G, g: Q4G. The left-hand I-bar represents the Bonferroni adjusted Fisher least significant differences between treatments (B) and the right-hand I-bar represents the Bonferroni adjusted Fisher Least Significant Differences within treatment comparisons (W). The bar through the zero time point indicates how far above or below is significantly different from it.

8.7.2 Digestion of pure polyphenolic compounds (controls) by the BB digestive phase in the absence of bile and pancreatin

The relative concentrations of the positive (Q3G) and negative (Q3R) controls, the derivative quercetin and the internal standard are shown in figure 32.

8.7.2.1 Epicatechin (internal standard) (control)

There were no significant differences in concentration between the BBMV and the HBBMV fractions for epicatechin in the control digest, and relative concentrations did not vary significantly over time.

8.7.2.2 Quercetin-3-glucoside (positive control)

Unlike Q3G in the onion extract, pure Q3G was readily deglycosylated by LPH during BB *in vitro* digestion. There was a large significant difference in the relative concentrations of Q3G between the fractions (d.f. 1,22, $F=220.36$, $p<0.001$). There was a significant time effect seen in the BBMV fraction with relative concentrations of Q3G significantly decreasing from 30-120 minutes (d.f. 3,22, $F=82.89$, $p<0.001$), and a significant time.BBMV fraction effect (d.f. 1,22, $F=91.22$ $p<0.001$).

8.7.2.3 Quercetin-3-rutinoside (negative control)

The relative concentrations of Q3R did not vary significantly between fractions and over time suggesting that Q3R was not deglycosylated by BB LPH.

8.7.2.4 Quercetin (control)

In the absence of active BB enzyme in the HBBMV fraction no quercetin was detected as expected. Although there were large increases in the relative concentration of quercetin in the BBMV fraction over time they were not significant (d.f. 3,11, $F=10.39$, $p=0.071$), but figure 32 that there was a steady increase in the relative concentration of the aquercetin, confirming Q3G hydrolysis.

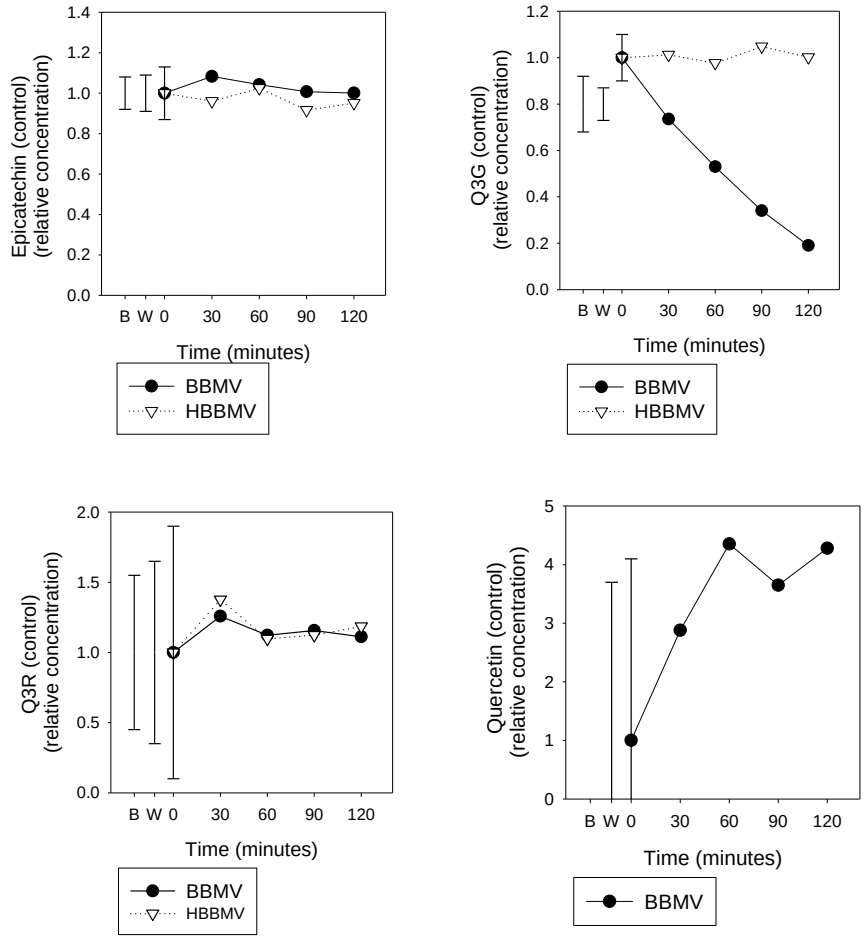

Figure 31. The relative concentration the internal standard epicatechin, and the purified polyphenolic glycosides (controls) present during a 2 hour BB *in vitro* digestion. Samples include active brush border enzyme (BBMV) and inactive (heat treated) BBMV enzyme (HBMV), n=3. The left-hand I-bar represents the Bonferroni adjusted Fisher least significant differences between treatments (B) and the right-hand I-bar represents the Bonferroni adjusted Fisher Least Significant Differences within treatment comparisons (W). The bar through the zero time point indicates how far above or below is significantly different from it.

8.7.3 Suitability of the BB digestive phase for the digestion of plant polyphenolic compounds

Susceptible polyphenolic compounds were suitably deglycosylated in the presence of active BBMV enzyme, and there were no significant changes in the relative concentration of those polyphenolic compounds in the presence of inactive enzyme. The BB digestive phase appears suitable for digestion of polyphenolic compounds.

8.7.4 Validation of the BB *in vitro* digestion method under conditions representing the small intestine.

After determining that the BB fraction readily deglycosylates susceptible polyphenolic glycosides, the method was ready to be tested under conditions representative of the small intestine, *i.e.* in the presence of biliopancreatic secretions in physiological concentrations. Figure 32 shows the concentrations (µg/ml) of Q3R and Q3G following BB digestion, the appearance of quercetin, and the concentration of the internal standard epicatechin.

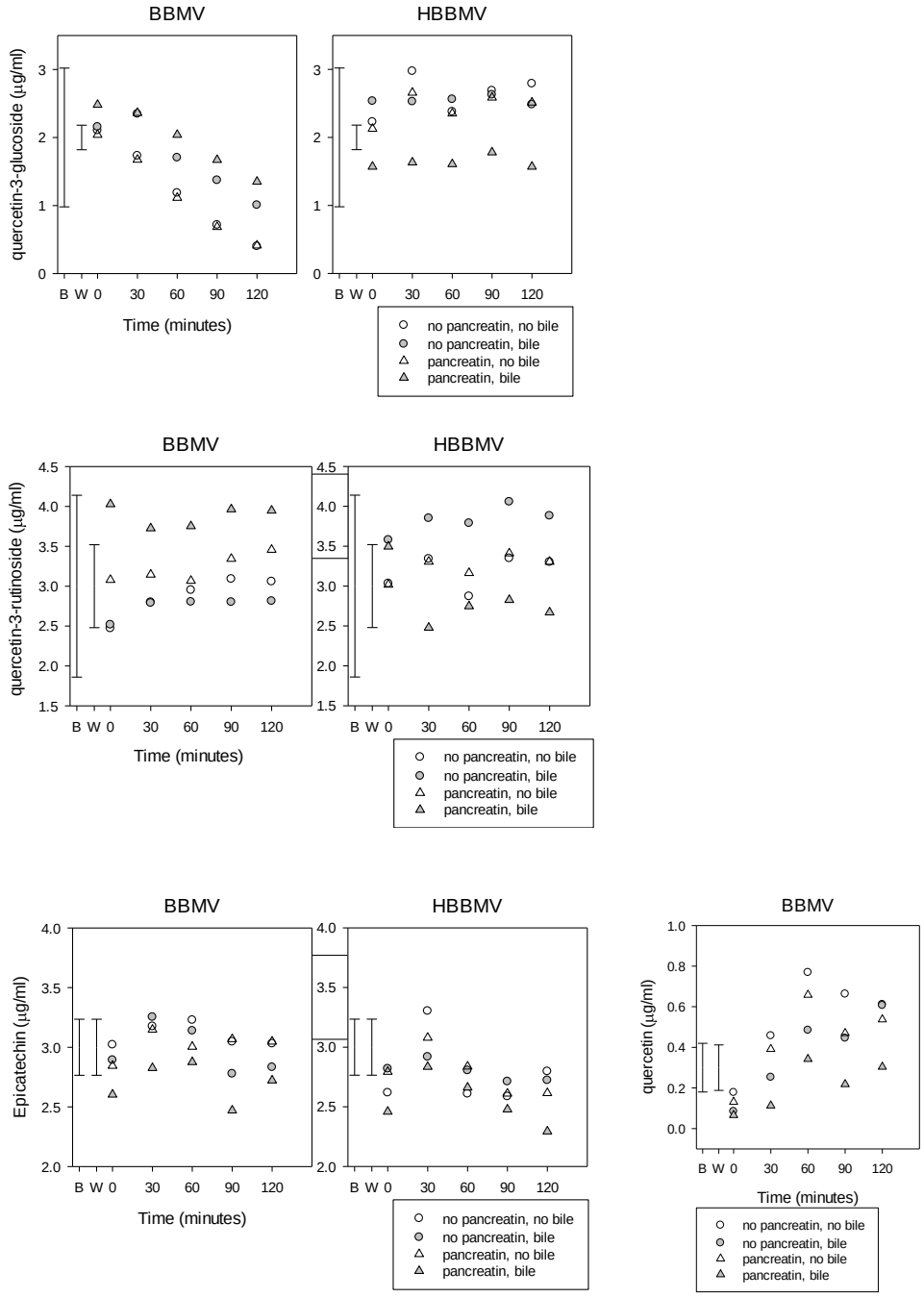

Figure 32. The measured concentration of epicatechin (internal standard), quercetin, Q3G and Q3R as determined by LC-MS. Samples include active brush border enzyme (BBMV) and inactive (heat treated) BBMV enzyme (HBMV), n=3. The left-hand I-bar represents the Bonferroni adjusted Fisher least significant differences between treatments (B) and the right-hand I-bar represents the Bonferroni adjusted Fisher Least Significant Differences within treatment comparison.

8.7.4.1 Epicatechin (internal standard)

When measured by LC-MS the concentration of epicatechin was different across treatments, and was higher in the BBMV treatments than in the HBBMV treatments (BBMV effect, d,f, 1, 16, $F=146.92$, $p<0.001$), and samples with pancreatin and bile had lower relative concentrations of epicatechin than those with no pancreatin or bile, pancreatin only or bile only (Pancreatin effect, d,f, 1, 8, $F=21.4$, $p<0.001$), (Bile effect, d,f, 1,8, $F=26.88$, $p<0.001$) or (Pancreatin x bile interaction, d,f, 1, 16, $F=21.4$, $p<0.001$) respectively. There was a strong Time effect (d,f, 4, 63, $F=20.68$, $p<.001$) but also a significant Time x BBMV and Time x BBMV x Bile interactions (d.f. 4,63, $F=2.88$, $p=0.037$ and d.f. 4,63, $F=3.05$, $p=0.029$ respectively). Both BBMV and HBBMV samples without bile had an increase in the relative concentration of epicatechin between 0 and 30 minutes, and the HBBMV samples without bile showed a drop between 30 and 60 minutes.

8.7.4.2 Quercetin-3-glucoside

All of the BBMV treatments show a significant decline in Q3G over time (Time x BBMV interaction d,f, 4,63, $F=197.23$, $p<0.001$), with the bile added treatments declining significantly less (Time x BBMV x bile interaction d,f, 4,63, $F=20.68$, $p<0.001$) than those treatments containing no bile (figure 32). There appear to be no significant differences over time for the HBBMV samples with bile; the HBBMV samples without bile are significantly higher (Time x BBMV x bile interaction d,f, 4,63, $F=20.68$, $p<0.001$) at 30, 90 and 120 minutes than at 0 minutes.

8.7.4.3 Quercetin -3-rutinoside

The quercetin-3-rutinoside was present as a negative control and was not expected to be hydrolysed by active BBMV enzymes. Two wells of data (for the HBBMV, pancreatin, no bile combination) were excluded as the values (zeroes) were suspect.

There were no significant time effects in the BBMV or HBBMV treatments; p value for Time effect was 0.131, and interactions involving Time had p values between 0.097 and 0.545 suggesting that there was no hydrolysis of quercetin-3-rutinoside by either active enzyme or by active biliopancreatic secretions in the heat treated BB sample (HBBMV). There appears to be a significant difference between the BBMV samples with and without pancreatin (BBMV x pancreatin interaction d,f, 1,14, F=5.81, p=0.030), but there are no time or bile effects.

8.7.4.4 Quercetin

The quercetin measured here is the metabolite formed by the deglycosylation of quercetin-3-glucoside by LPH during BB *in vitro* digestion. Since, there was no active LPH in the HBBMV fraction no quercetin aglycone was found.

When bile (Bile main effect, d,f, 1, 8, F=19.39, p=0.002) or pancreatin (pancreatin main effect, d,f, 1, 8, F=42.35, p<0.001) were present the appearance of quercetin was reduced compared to BBMV alone, and these actions occurred independently (figure 32). Aglycone levels increase over the first 60 minutes (Main time effect, d,f, 4, 53, F=111.07, <0.001), but the increase is significantly smaller if bile is added (Time x bile interaction, d.f. 4,53, F=7.14, p=0.16).

8.7.4.5 Summary

Although bile and pancreatin caused fluctuations in the relative concentrations of the polyphenolic compounds measured by LC-MS the most important finding was that the

proposed BB *in vitro* digestion methodology functions appropriately in conditions representative of the small intestine.

8.8 Discussion

The only endogenous β-glycosidase present in the lumen of the small intestine is LPH. Although other β-glycosidases are present in enterocytes, the liver and associated with bacteria resident in the large intestine (Walle, 2004). In the small intestine LPH has a preference for hydrophilic substrates (Day et al., 2000), but hydrolyses a variety of β-glycosides including lactose and polyphenolic compounds (Day et al., 2000, Nemeth et al., 2003a). Polyphenolic compounds are hydrolysed to an aglycone and a glycoside (Day et al., 2000) in a process that is dependent on the configuration of individual polyphenolics. Lactase-phlorizin hydrolase's affinity for specific glycosides is dependent on the hydrogen bonding of 5 amino acids present at the active site and for hydroxyl residues present on the substrate (Marana, 2006). The orientation of these hydroxyl groups on the polyphenolic glycosides is also important (Fernandez et al., 1995). Hence, some but not all polyphenolic glycosides will be susceptible to BB LPH hydrolysis.

8.8.1 The deglycosylation of polyphenolic compounds

As there is interest in the digestion, metabolism and absorption of polyphenolic glycosides they have been extensively studied. Of the quercetin glycosides digested here by *in vitro* BB digestion Q4G, I4G and Q3G, but not Q3R or Q3,4DI were hydrolysed. Previous works confirm that Q4G (Arts et al., 2004, Day et al., 2000, Nemeth et al., 2003b, Ioku et al., 1998), and Q3G (Arts et al., 2004, Day et al., 2000, Nemeth et al., 2003b, Ioku et al., 1998) are hydrolysed by LPH. Information was not found on I4G deglycosylation. Other reports confirm that Q3R (Day et al., 2000, Nemeth et al., 2003b)

and Q34G are not deglycoyslated by LPH (Day et al., 1998), but others suggest Q3R is nominally hydrolysed (Ioku et al., 1998).

The interesting finding was that purified Q3G (positive control) was readily hydrolysed during *in vitro* BB digestion, but onion extract Q3G was not hydrolysed. It has been suggested that Q3G and Q34DI may not fit into the active site (Day et al., 1998), but as purified Q3G was hydrolysed this may not be the case. The concentration of Q4G and I4G in raw onion are 30 and 4 fold greater than Q3G respectively (Neveu et al., 2010). So when Q3G is in the presence of other polyphenolic glycosides substrate competition means that Q3G may not be favoured.

The rate at which polyphenolic glycosides are hydrolysed may also influence their relative concentration during BB digestion. For example the deglycosylation of Q4G occurs at twice the rate of Q3G (Arts et al., 2004), although over the course of a 90 minute digestion others have found that the rate of Q4G hydrolysis is 5.5 fold greater than that of Q3G, *i.e.* 90 µmol/min/mg *vs.* 16.2 µmol/min/mg repectively (Day et al., 1998). Hence, in the lower substrate concentrations and with the slower hydrolytic rate Q3G hydrolysis may not be high.

Although the relative concentration of Q34DI was significantly lower in the BBMV fraction there was no evidence of its hydrolysis. This is in contrast to a report showing that 1) Q34DI can be hydrolysed fully to its aglycone and glucosides by LPH and 2) that the 4′ glucoside of Q34DI can be hydrolysed resulting in Q3G and glucose (Nemeth et al., 2003b). If this occurred, in this work, the concentrations of Q34G would have declined during digestion, and Q3G would have increased, which they did not. Further examination using labelled glycosides would be needed to confirm this.

In addition to the production of Q3G from Q34DI hydrolysis, another option to explain the lack of Q3G hydrolysis in the onion extract may be that the Q3G aglycone is

susceptible to transglycosylation in situations where there are large concentrations of product (glycosides); oligosaccharide hydrolases of the glucosyl 31 family can transglycosylate in such situations of excess product (Dahlqvist, 1960a, Zagalak and Curtius, 1975). Again more investigation may be required. Others suggest that Q3G and Q34DI may bind with LPH to form a non-productive complex (Day et al., 1998). This non-productive complex may be the covalent intermediate formed in the deglycosylation process (Marana, 2006). This could account for the lack of hydrolysis seen in Q3G and Q34DI during hydrolysis.

8.8.2 The fate of polyphenolic compounds during *in vitro* digestion with biliopancreatic secretions

The large degree of variation of the relative concentration of quercetin compounds in section 8.7.2, compared to section 8.71, suggests that biliopancreatic secretions may interfere with either the hydrolysis or measurement of polyphenolic compounds under BB *in vitro* conditions. Other factors such as degradation or oxidation may be also be involved. Following biliopancreatic digestion there is a decrease in anthocyanin (Pérez-Vicente et al., 2002) and flavonols concentrations (Vallejo et al., 2004). For anthocyanins this may result from the transformation of the flavylium cation into a chalcone (Pérez-Vicente et al., 2002), but a another possibility is that the anthocyanins form insoluble complexes with biliopancreatic components (McDougall et al., 2005) or other components of the digestate (Vallejo et al., 2004).

In this work there was a slow development of a yellow colour during the course of the digestion, which increased after the addition of the BBMV aliquot and the pH adjustment (from 6 to 8) at 1 hour. It was most prevalent in the onion extract. A search of the literature found that there is a shift in wavelength when quercetin binds to proteins such as albumin, resulting in the absorbance of Q3R (363nm) and quercetin (375nm) shifting to a longer

wavelength of 410nm (Manach et al., 1996). Hence, binding of the proteinaceous component of the BBMV fraction to polyphenolic compounds may be the cause of some of the yellow colour development.

Another possibility is a colour change due to changes in pH. Flavonoids can undergo chemical modification in aqueous solution at alkaline pH due to auto-oxidation of the C-ring that causes a shift in wavelength (Jurasekova et al., 2014). Flavonols and flavon-3-ols are susceptible to mild alkaline conditions and may change into unknown or undetected forms (Bermúdez-Soto et al., 2007). Further, quercetin may form complexes with metal ions, such as manganese forming coloured complexes (Bravo and Anacona, 2001). Hence, in the presence of bile and pancreatin analysis of polyphenolic compounds can be problematic. These results could explain the high level of variability seen in these results.

The onion extract contained only trace amounts of quercetin, yet at 0 hours there was a large amount of quercetin in samples. There are three possible explanations; the first that deglycosylation occurred rapidly and was subsequently measured, the second that the addition of 50 % methanol (post sampling) did not stop the LPH activity, and thirdly, since BBMV enzymes, including LPH, are stable when frozen for a year (results not shown) once defrosted they may continue to hydrolyse polyphenolic compounds. Other enzymes are stable under these conditions so this seems plausible; for example leucine aminopeptidase is stable for 25 hours in the presence of 50 % methanol and continues to be active at sub-zero temperatures (Lin and Van Wart, 1982). Further investigation is required.

Quercetin is solubilised by bile salts, but at low pH levels it may become less soluble, but when the pH increases bile's affinity for quercetin increases (Buchweitz et al., 2016). So the quercetin may partition into the micellar phase, which may make the substrate less

accessible to LPH. Further, as mentioned previously, the membrane surface of the BBMV may act as a solubilising surface for hydrophobic substrates (Zhou and Schulten, 1996), but when bile is attached to these substrates it may sterically hinder this process, which may hinder hydrolysis.

8.8.3 Suitability of the adjunct BB phase of digestion for the digestion of polyphenolic compounds

Although large variations in polyphenolic digestion are seen when bile and pancreatin are present this does not detract from the fact that the BB *in vitro* digestion method operates under the small intestinal conditions. The ability for this system to function under non-physiological conditions would need to be determined for those wishing to use it as an adjunct to existing *in vitro* methodologies.

8.8.3.1 Why is the BB *in vitro* digestive phase important?

Research indicates that polyphenolic compounds, particularly the aglycones, interact with bitter taste receptors of the small intestine and induce signalling cascades that initiate satiety (Roland et al., 2011, Rozengurt, 2006, Jang et al., 2007). This involves the triggering of neurological receptors that induce an enteroendocrine response (Rozengurt, 2006). The physiological response initiates satiety, and as such the ingestion of potentially toxic food sources. Being that polyphenolic compounds are xenotoxic it is likely that they are rapidly detoxified quickly by phase I and II metabolism (Meunier et al., 2004, Scalbert and Williamson, 2000). So in order to be affective appetite suppressants polyphenolic aglycones would need to be effective in low concentrations or present in high concentrations at the intestinal microvillar membrane. Polyphenolic compounds are generally found in a glycosylated form in plants, and as evident here not all polyphenolic compound are hydrolysed by LPH. As such, a functioning system such as the adjunct BB *in vitro* digestion system may be able to identify those polyphenolic compounds that are

hydrolysed by BB LPH, and provide aglycones at concentrations likely initiate strong satiety responses. This may lead to the finding of novel bioactive compounds with nutraceutical potential.

The next step in the validation of the *in vitro* BB digestion method would be to digest proteins and carbohydrates. These experiments were in the planning stage, but could not be accomplished within the time limits of a thesis. *Beta*-limit dextrin would be a suitable substrate for studying the influence of BB enzymes on carbohydrate digestion, as it is the carbohydrate remaining following pancreatic digestion. Bovine serum albumin would be an appropriate substrate for oligopeptidases as it is well characterised and is a relative small protein. Initial concentrations could be chosen based on the proportion of CHO and protein present in the "meal" that was integral to development of the BB *in vitro* digestion phase based on all fluid volumes seen in the digestive tract (section 4.3). However, there is scope to examine many other aspects of digestion not previously investigated.

8.9 Conclusion

Physiological systems are fraught with variation, and this system was no different. However, all of the evidence so far suggests that the adjunct BB *in vitro* digestion method may be a useful tool in the ongoing investigation of the nutrient and non-nutrient components of food.

9 Chapter 9. Discussion

This thesis presents a novel approach to human *in vitro* digestion with the development of a validated BB *in vitro* digestion phase that can be used as an adjunct to current *in vitro* digestion methods. To my knowledge this is the first time anyone has utilised a replete consortia of BB enzymes for the *in vitro* digestion of polphenolic compounds. However, different iterations of a BB digestive phase have been used in the digestion of proteins for some time (Shan et al., 2002, Hausch et al., 2002, Picariello et al., 2010, Gianfrani et al., 2015, Mamone et al., 2015, Picariello et al., 2016) A recent review into the use of BBMV to stimulate human intestinal digestion (Picariello et al., 2016) suggests that there needs to be a consensus on conditions such as pH, substrate:enzyme ratios and incubation times, as well as the developed method being validated. It is hoped that the findings of this thesis will bring this field closer to producing a realistic BB *in vitro* digestion model. Regarding the BB model, the INFOGEST group https://www.cost-infogest.eu/ have recently signalled an interest in the incorporation of a BB phase of *in vitro* digestion to the INFOGEST method (Minekus et al., 2014).

9.1 Development of an adjunct BB *in vitro* digestion method

This research had three main aims. Firstly, to identify points within the mammalian alimentary tract where *in vitro* methods could be better aligned with *in vivo* digestion. Secondly, to develop and validate a consortium of enzymes that emulate intestinal BB digestion in an *in vitro* system. Thirdly, to then assess digestion of polyphenolic compounds using the BB *in vitro* method. A discussion on aspects of the development of the adjunct BB *in vitro* digestion will be under headings based on the three aims, and will

focus primarily on results that affected the development of the BB *in vitro* digestion method.

A review into current methods of *in vitro* digestion (**Chapter 1**) revealed that the most important way to align *in vitro* with *in vivo* digestion was to include a BB phase as an adjunct to current methods of small intestinal digestion. A review of the secretion and action of BB digestive enzymes **(Chapter 2)** demonstrated that the BBMV incorporate a large array of 103 digestive hydrolases (McConnell et al., 2011) that would be difficult to mimic. Due to the need for the BB *in vitro* phase to be physiologically relevant it was decided that *ex vivo* mammalian BB enzymes should be utilised to ensure the relevant array of enzymes were represented. A BBMV preparation was isolated from rat mucosal scrapings, and the preparation was found to be a stable, economical and efficient way to provide a consortia of BB enzymes for *in vitro* digestion. The activities of BB enzymes against model substrates were assessed under conditions representative of the small intestine, *i.e*. in the presence of physiological ratios of bile and pancreatic enzymes, under pH conditions representative of the small intestine, over time, with changes in pH, and upon solubilisation **(Chapters 6 and 7).** This guided the development of the *in vitro* BB phase of digestion. Once the BB *in vitro* methodology was resolved differences in the kinetic activities of enzymes between BBMV preparations were investigated using the buffer and pH conditions chosen for the *in vitro* digestion **(Chapter 5)**, and then the BB phase was validated using selected polyphenolic compounds as substrates **(Chapter 8).** From this body of work there were several significant research finding that are described below, and in figure 33.

9.2 Identification of points within the alimentary tract where *in vitro* models could better represent the *in vivo* process

The first aim of this thesis was to identify ways that current methods of *in vitro* digestion could be better aligned with the physiological process of digestion. In order to do so an understanding of the kinetics of digestion from the mouth to the terminal ileum is required. By understanding the process of digestion one can identify and prioritise areas in which current *in vitro* digestion models could be brought closer in line with the physiological process of digestion.

The digestive processes were examined from the mouth to the terminal ileum, in the context of how these systems are integrated into current methods of *in vitro* digestion. *In vitro* methods vary from simple static methods that emulate aspects such as enzymes, pH and the physicochemical environment (Minekus et al., 2014), to complex systems that control aspects of digestate transit, the secretion of biological fluids and the physical processing of food. Complex models include the TIM-1 model (Verwei et al., 2006), the DGM (Wickham and Faulks, 2012) and HGS (Kong and Singh, 2010). By assessing these systems in the context of physiological relevance we can begin to identify what factors are limiting and therefore could be improved.

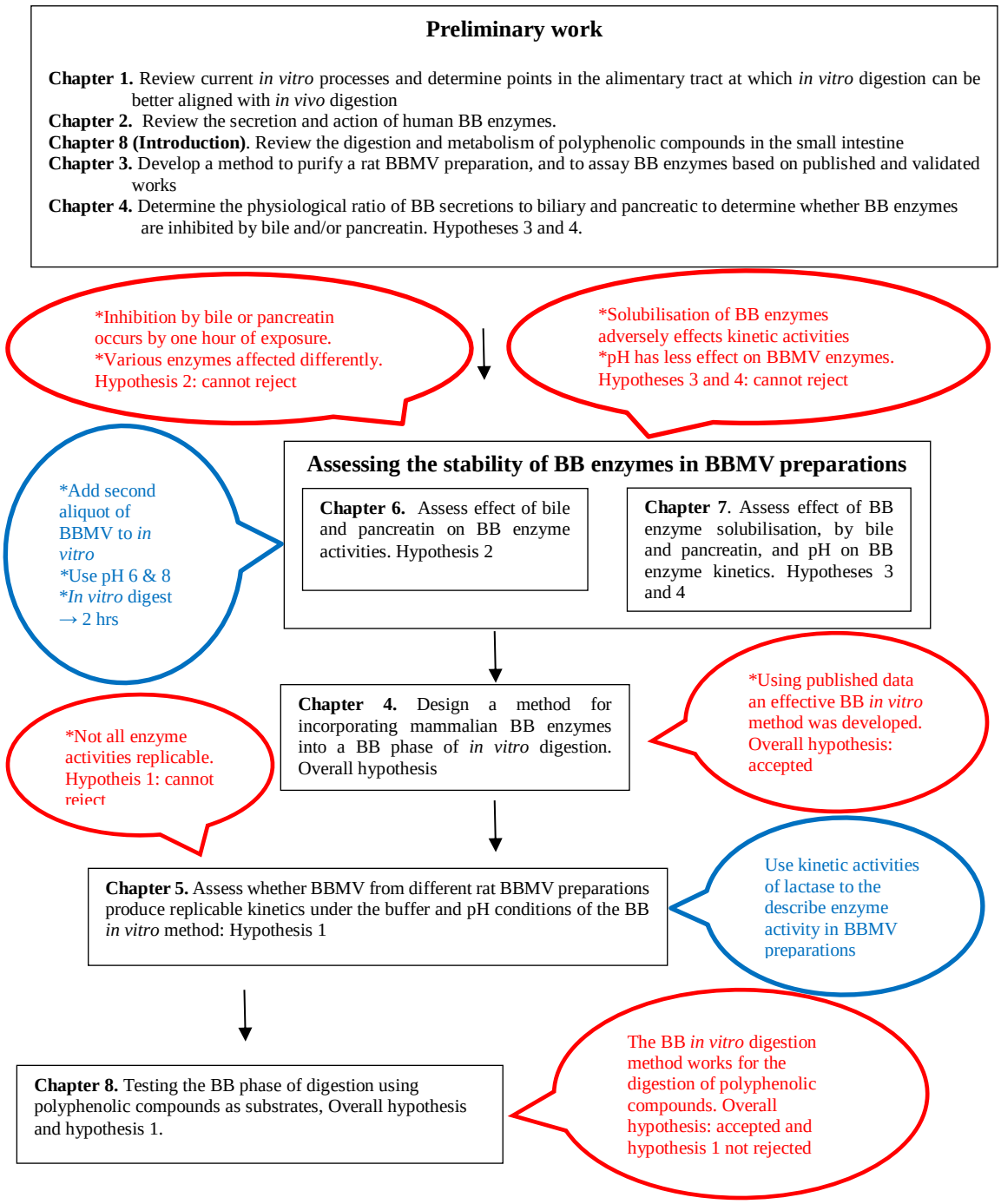

Figure 33. Schematic showing the flow of experimental work along with key findings affecting the development of the *in vitro* BB phase of digestion
Red text boxes: indicate findings; Blue text boxes: responses to key findings

Points identified for further investigation include, but were not limited to, allowing the food boli to remain intact in the gastric chamber prior to initiating the motile phase,

monitoring and adjusting the pH of the gastric phase, using physiologically relevant enzyme concentrations, using forces and kinetics in keeping with those found *in vivo,* and using replete enzyme arrays in each digestive phase. Ensuring all the appropriate enzymes are present in each digestive phase may be of particular importance as it has been shown that the presence of peptidases in the *in vitro* gastric phase can increase the rate of lipid hydrolysis in a milk formula emulsion (Lueamsaisuk et al., 2013). A significant omission from the most current *in vitro* methodologies is the absence of a replete BB phase of digestion. Brush border enzymes have an important role in the terminal digestion of food, and their absence can cause malnutrition (Holzinger et al., 2002, Tsai and Duggan, 2005), and varying degrees of lower digestive tract disease and distress (Tsai and Duggan, 2005). So being able to model terminal digestion in the laboratory could improve current understanding of terminal digestion.

It would be satisfying to implement all the improvements suggested here, and in **chapter 1**, but that was beyond the scope of this thesis. Instead this body of work aimed to remedy the current omission of a BB phase of digestion by developing a physiologically relevant BB *in vitro* digestion method that can follow the biliopancreatic phase of *in vitro* digestion.

9.3 Simulating intestinal brush border digestion in an *in vitro* system

The second aim of this thesis was to develop and validate a consortium of enzymes that would emulate intestinal BB digestion in an *in vitro* system. For an enzymatic system that is often overlooked during *in vitro* digestion, enzymes of the small intestinal BB provide a wide range of digestive enzymes that have the ability to terminally digest nutrient oligomers prior to absorption **(Chapter 2).** The microvillar membrane is also a complex biomechanical organelle that vesiculates and sheds BBMV, along with active BB

enzymes, into the lumen of the small intestine (McConnell et al., 2009). A method for harvesting these BBMV (calcium precipitation and differential centrifugation) was developed and validated in the 1970's (Schmitz et al., 1973, Kessler et al., 1978), providing us with a convenient method to source a discrete consortium of enzymes that could be used to develop a method for *in vitro* BB digestion. Brush border enzymes are integrated into BBMV membranes, which provide a scaffold of support and stability (Hooton et al., 2015), and an initial series of experiments showed that BBMV enzymes are relatively stable for at least a year when stored at -80°C, and can even remain active at room and fridge temperature (appendix 4).

In the human duodenum the osmolality, ionic strength and concentration of bile acids vary significantly between humans in the fasted state (Lindahl et al., 1997). Likewise the volume of pancreatic juices varies depending on nutrient intake, body weight and time (Holtmann et al., 1996). The secretion and action of BB enzymes are no different. Brush border enzyme activities vary with diet (Tanaka et al., 2008, Goda et al., 1995, Ferraris et al., 1992), life-stage (Fan et al., 2002), and circadian rhythms (Saito et al., 1976, Stevenson et al., 1975). The initial series of experiments showed that there were large inter-individual differences in digestive enzyme activities, so in the course of this study diet and husbandry were standardised so as to minimise the affect on the activities of BB. One of the main steps was to pool the mucosae of animals for each experiment to minimise variation. Inevitably there were still variations, but these factors did not preclude its use to bring current methods of *in vitro* digestion more closely in line with *in vivo* digestion.

In order to determine whether the BBMV harvested from mammalian mucosal tissue was viable for use in an *in vitro* digestion system BB enzymes were characterised, and a rationale was developed for the development of an *in vitro* digestive phase. Published

human data were used to determine the physiological ratios and concentrations of exogenous (dietary intake), and endogenous fluids (buccal, gastric, pancreatic, biliary, Brunner's gland, and *succus entericus* secretions) contributing to the fluid load in the small intestine at 1 hour post-prandial. Published physiological data (**Chapter 4**) was also used to determine at what concentration bile and pancreatin would be present during an *in vivo* digestion, and these were added in these ratios to an *in vitro* digestion. As mentioned in **Chapter 1**, many *in vitro* digestion methodologies do not use physiological concentrations of bile and pancreatin, which may alter the range of digestive derivatives from *in vitro* digestion. This is evident in **Chapter 6** where differences in pancreatin concentration altered the activities of BB oligosaccharidases. In the temporal assays the concentration of pancreatin in the final reaction vessel was 1.2- fold less than for the exposure assays, this dramatically inhibited the activities of maltolytic enzymes. Some *in vitro* models, *e.g.* (Pitino et al., 2010) use a concentration of amylase (in pancreatic secretions) that was 11 fold greater than present *in vivo* (Ulleberg et al., 2011), and because these authors do not account for all small intestine fluid volumes the final concentration of amylase in their small intestinal digest was still 3.3 fold greater than the concentration of amylase in pancreatic juice. It is difficult to know whether such a large increase in pancreatin improve or encumber enzyme activities, but this may be an obstacle for use with the BB *in vitro* digestion method, and methodological modifications may be required.

The *in vitro* rationale was tested in several ways. A representative group of enzymes were assessed kinetically to determine the reproducibility of enzyme activities in the BBMV preparations processed from different groups of rats **(Chapter 5).** In the presence of biliopancreatic secretions **(Chapter 6)** and following bile or pancreatin solubilisation, and at a range of pH's **(Chapter 7)**.

9.3.1 Different preparations of BBMV differ in enzyme kinetics

In **Chapter 5** it was shown that these results were in keeping with those previously reported in the literature; although different BBMV preparations resulted in statistically different kinetic activities, with significant differences in enzyme K_m and $V_{max.}$ A comparison of kinetics will not be discussed here as this was covered **chapter 5**, but the null hypothesis that the kinetic activities of BB enzymes from different preparations of rat BBMVs are not replicable could not be rejected.

In order to keep experiment to experiment variations to an absolute minimum the same laboratory equipment was used throughout and all tissue sampling, BBMV preparation, and enzyme assays were performed by the author. A possible explanation for the high level of variation may be the way the data was processed. Variations in enzymatic activities at each substrate concentration were all relatively similar. However, as the method of analysis calculates kinetic values from all of the points on the sigmoidal curve the degrees of freedom are high. The values are not true mean values rather they are calculated values with a measurement to show the degree of error. No sum of squares were available and the conventional ANOVA method could not be used, although an alternate was investigated (Cohen, 2002).

The high degree of variability meant that an alternative method to define the amount of enzyme activities in the BBMV preparation was required. The method chosen treated BBMV preparation much like pancreatin preparations. Pancreatin is prepared in terms of the amount of pancreatin required to achieve certain levels of α-amylase, trypsin, and lipase activities, seen *in vivo*, *i.e*. *x* amount of pancreatin will supply *x* amount of activity (of α-amylase, trypsin or lipase) under certain conditions. From data obtained in lactase activities were the most replicable, and have been proposed as the measure of probable activity under certain physiochemical conditions. For example a 40 µl aliquot of BBMV

preparation (equivalent to 2 mg/ml of the starting mucosal homogenate) provides 134 - 142 pmol/min of lactolysis under the conditions of 10 mM phosphate buffer (divalent cations, 50 mM mannitol) pH 6, 37°C, and 60 minutes. Hence, enzyme concentrations can be calculated to ensure adequate hydrolysis. In future work these concentrations could be adapted to suit experimental needs, but caution should be taken to make sure that substrate concentrations suit the kinetic capabilities of the enzyme of interest.

9.3.1 Biliopancreatic secretions significantly affect the activities of BB enzymes.

After initial characterisation, an array of BB enzymes were assessed for their abilities to function in the environment of the *in vitro* digestion. There was an absolute requirement that the *in vitro* BB phase of digestion functioned under physiological concentrations of biliopancreatic secretions. It was evident from the results that the different BB enzymes had varying reactions to biliopancreatic secretions **(chapter 6),** table 47 and 48**,** being either activated, inhibited or unchanged by the presence of bile and/or pancreatin compared to in the absence of biliopancreatic secretions. In general bile and pancreatin significantly inhibited BB hydrolysis under conditions representative of the human small intestine so the null hypothesis that rat BB enzymes will be significantly inhibited by the presence of bile and pancreatic enzymes could not be rejected. This was not unexpected as the role of bile and pancreatin is the denaturation and degradation of nutrients.

There was a 1.2-fold increase in pancreatin between the temporal and exposure assays, which saw a large increase in maltolytic activities. This could suggest that the pancreatic α-amylase is augmenting maltolytic activivity in the exposure assay. The change in method also resulted in a 4.9 fold increase in bile, but large differences in the activities of enzymes assayed in both experiments were not obtained. This suggests that for *in vitro*

methods wanting to incorporate a BB *in vitro* phase of digestion to their current methods the concentration of bile and pancreatin may be less of an issue than first thought.

Table 47. The overall effects of bile and/or pancreatin on enzyme activities in the temporal assay (Chapter 6)

Enzyme	Fraction	Pancreatin	Bile	Pancreatin plus bile
Maltase	BBMV	-	↓	↓
	Aqueous	-	-	↑
ALP	BBMV	↓	-	↓
	Aqueous	↓	↓	-

Table 48. The overall effect of bile and/or pancreatin on enzyme activities in the exposure assay (Chapter 6)

Enzyme	Fraction	Pancreatin	Bile	Pancreatin plus bile
Lactase	BBMV	–	↓	-
Maltase	BBMV	↑↑	–	↑↑
Glucoamylase	BBMV	↑↑	–	↑↑
Sucrase	BBMV	–	↓	-
Isomaltase	BBMV	–	↓	↓
NEP	BBMV	↑↑	↓↓	↑↑
APN	BBMV	↓	↓↓	↓↓↓

The important lesson from **chapter 6** was that each enzyme remained functional following *in vitro* digestion under biliopancreatic conditions, but for some (like APN) the combination of bile and pancreatin was deleterious. As a result a second aliquot of BB enzyme would be added to future *in vitro* digestion to supplement those enzymes that were significantly inhibited by bile and pancreatin. By including additional BB enzymes the proposed BB phase would better emulate the continued secretion and action of BBMV and the associated enzymes, along the length of small intestine (Hooton et al., 2015).

The *in vitro* digestion of plant polyphenolics **(Chapter 8)** included the addition of a second aliquot of enzyme. There did not appear to be any significant change in the

deglycosylation rate of polyphenolic glycosides suggesting that although the BB enzymes had been replenished, the increase in pH from 6 to 8 may have inhibited lactase, which is in keeping with what is reported in **chapter 7.** There was a 3.5 - fold decrease in lactolysis when the pH of the reaction increased from 5.6 to 7.6. Hence, the change from pH 6 to 8 during the *in vitro* digestion would likely result in a slowing in the deglycosylation rate. In contrast the V_{max} of maltase and sucrase activities were increased at pH 7.6.

Further investigation **(Chapter 7)** of the effects of biliopancreatic secretions showed that BB enzymes are solubilised by bile and pancreatin and that this process can alter an enzymes ability to cope with changes in pH further supporting the need for a second aliquot of enzyme. None of the previous BBMV *in vitro* digestions used a second aliquot of enzyme (Shan et al., 2002, Hausch et al., 2002, Picariello et al., 2010, Gianfrani et al., 2015, Mamone et al., 2015, Picariello et al., 2016), but given these results it may be an important development in the method

9.3.1 The solubilisation of BBMV-bound BB enzymes results in variants with different kinetics to BBMV-bound enyzmes

It has been previously shown that bile and pancreatic enzymes solubilise BB enzymes from the surface of BBMV (Maestracci, 1976, Vasseur et al., 1978, Young and Das, 1990), but this is the first time that has been assessed over different pH levels. Gastric contents enter the small intestinal at pH 2-5, and the pH changes during aboral transit from approximately 5 in the proximal duodenum to 8 in the ileum (Oomen et al., 2002). In addition to gross changes in the luminal pH BB enzymes can also be subjected to changes in pH due to pH microclimates within the small intestine. For example, there is thought to be an acidic milieu surrounding BBMV due to active sodium/proton antiporters (Murer et al., 1976, Ganapathy et al., 2006), and at the BB surface there may be an alkalinised microclimate surrounding alkaline phosphatase (Mizumori et al., 2009).

Hence, assessing BB enzymes and their solubilised variants over a succession of pH levels is important to the understanding of their action *in vivo*.

Although BB enzyme solubilisation could be an efficient way to disperse BB enzymes through the luminal contents, significant differences in enzyme kinetics upon solubilisation meant that they may not be as effective as BBMV-bound enzymes. The most significant finding was that the loss of the transmembrane segment, during proteolytic solubilisation, appears to significantly decrease substrate affinity and reduce maximal activity. This loss of activity has been observed in previous studies (Young and Das, 1990, Maestracci, 1976). The altered kinetic activities upon solubilisation could explain some of the lost activities seen in **chapter 6**. However, it is likely that there was some proteolysis and denaturation as well (Jones, 1992, Semenza, 1986). This is of particular interest in the case of lactase. It appeared that no lactase was solubilised by pancreatin, and that bile solubilisation was only effective at pH 5.6 and 6.6, *i.e.* close to the pH optimum. Since pancreatin alone did not significantly inhibit lactolysis it may be that lactase was not readily soluble under these conditions; although other work suggest it is and readily loses activity upon solubilisation (Young and Das, 1990). It may be that solubilised variants are susceptible to changes in tertiary structure following solubilisation.

The kinetic activities of the solubilised variants were more readily affected by changes in pH compared to the BBMV-bound enzymes, which supports the need to have a BB phase that allows for the functioning of both the oligosacharidases and the oligopeptidases. Interestingly at pH 7.6 sucrase and maltase activities were high suggesting that oligosaccharide hydrolysis would continue during the second hour of digestion. The primary finding here was that solubilisation alters the maximal activity of BB enzymes, and provides further evidence for the need for a second aliquot of enzyme during *in vitro*

digestion. In this case we cannot reject the null hypothesis that rat BBMV-bound enzymes will have significantly different enzyme kinetics to those that have been solubilised from the small intestine by bile or pancreatin.

9.3.2 The effect of bile and pancreatin solubilisation on BB enzymes across pH compared to BB-bound enzymes

The primary finding in **chapter 7** was that BBMV-bound enzymes tended to be stable across pH, but their solubilised variants were not. There was a general loss of activity with increasing pH for pancreatin solubilised and bile solubilised enzymes suggesting that the BBMV does indeed provide enzyme stability (White and Wimley, 1998). Hence, the null hypothesis that the kinetic activities of bile and pancreatin solubilised rat BB enzymes will vary significantly due to changes in pH compared with rat BB-bound enzymes is accepted.

The important lesson here is that increases in pH exacerbate the negative effect of solubilisation by bile and pancreatin, generally resulting in reductions in maximal activities. Ensuring pH is physiologically appropriate is an aspect of *in vitro* digestion that must be considered as the pH of the small intestine fluctuates. We must ensure that both the oligosaccharidases and the oligopeptidases can function under the given conditions.

The *in vitro* BB method was designed not just for the hydrolysis of model substrates and polyphenolics, but for improving the fidelity of any *in vitro* digestion. Since oligosaccharidases function best around pH 6 (range 5.5 – 6.5) (Kolínská and Kraml, 1972, Sasajima et al., 1975, Sorensen et al., 1982, Skovbjerg et al., 1982) while oligopepidases function best at pH levels near their optimums around pH 8 (range 6.5 - 8.5) (Danielsen et al., 1980a, Danielsen et al., 1980b, Kozak and Tate, 1982, Caporale and Troncone, 1988, Yoshioka et al., 1987, Matsumoto et al., 1995) incorporating a pH

change during the BB *in vitro* digestion was important. The specific activities of most oligosacchridases and oligopeptidases appear to peak in the jejunum (Hooton et al., 2015) where the pH is reaching 7-7.5 (Oomen et al., 2002), suggesting that oligosaccharidases may be more active while attached to BBMV, where the pH may be slightly acidified, while oligopeptidases may function better near the surface of the small intestine where ALP alkalinises the microclimate (Daniel et al., 1989). Hence, the first hour of the BB *in vitro* digestion would be at pH 6 and would be buffered to 8 at 1 hr, with the addition of the second aliquot of BBMV preparation. In contrast a pH of 7.2 has been used in previous BBMV *in vitro* digestions for the hydrolysis of oligopeptides (Picariello et al., 2010, Mamone et al., 2015, Gianfrani et al., 2015), and are incubated for 4 (Mamone et al., 2015) to 6 hours (Picariello et al., 2010, Gianfrani et al., 2015) in contrast to 2 hours.

9.4 Assessing the digestion of polyphenolic compounds using the BB *in vitro* method

The third aim of this thesis was to assess the digestion of polyphenolic compounds using the BB *in vitro* digestion method. There has been a lot of work examining the digestion of foods or polyphenolic extracts containing polyphenolic compounds (table 44, section 8.3) with none of them using a complete small intestinal digestion (digestion that includes an *in vitro* BB phase). The absence of a BB phase of digestion is particularly important when looking at the impact of digestate containing polyphenolic compounds on cell integrity (Tagliazucchi et al., 2010, Laurent et al., 2007). Without deglycosylation by BB LPH all of the plant polyphenolic glycosides will still be present post-digestion, and any impact on cellular processes may be underestimated. The majority of polyphenolic compounds found in nature are in a glycosylated form (Bravo, 1998), but it tends to be the aglycones arising from LPH deglycosylation that have greater antioxidant potential than their glycosylated counterparts, and have greater capacity for limiting lipid oxidation

(Williamson et al., 1996). In **chapter 8** it was demonstrated that polyphenolic glycosides were resistant to pancreatic digestion, but were generally hydrolysed by the β-glycosidase (LPH) present in BBMV preparations. The polyphenolic compound quercetin 3,4 diglucoside was not hydrolysed during complete small intestinal *in vitro* digestion, which is in keeping with observations on the specificity of human LPH for quercetin glycosides (Day et al., 1998). Likewise the positive (Q3G) (Arts et al., 2004) and negative control (Q3R) (Manach et al., 1997) were susceptible and resistant to hydrolysis respectively. It is important to note that it is normal for polyphenolic glycosides to bypass BB digestion as some are deglycosylated in the enterocyte (Berrin et al., 2003), liver (Day et al., 1998) or colon (Olthof et al., 2003, Aherne and O'Brien, 2002). Showing that we can accept the alternate hypothesis that a preparation of rat BBMVs can be used to simulate the BB digestion of polyphenolic compounds under conditions representative of the human small intestine.

Polyphenolic glycosides, rich in the onion flesh extract, were readily hydrolysed under conditions representative of the small intestine. The relative concentration of onion flesh polyphenolic compounds were assessed by LC-MS, and showed that Q4G and I4G were readily hydrolysed but Q3G and Q34DI were not significantly hydrolysed during BB digestion. In contrast, the control Q3G (purified compound) was readily hydrolysed, suggesting that when favoured polyphenolic compounds are in excess competition for LPH may reduce the hydrolysis of polyphenolics with glycosides at the 3 position. The most common binding site on flavonols in nature is the 3 position (Herrmann, 1976) so the distinct lack of Q3G deglycosylation is an interesting finding. Previous work demonstrates that Q3G may be a less favoured substrate (Day et al., 1998). The initial rate of Q4G deglycosylation was 15 fold greater than Q3G, with 90 % and 16 % deglycoslyated after 90 minutes respectively (Day et al., 1998). It is reasonable to assume

that in admixture with other polyphenolics the lower specificity of LPH for Q3G may limit its hydrolysis. However, other comparable research suggests that ovine LPH displays similar substrate specificity and catalytic efficiency towards Q4G and Q3G respectively (44μm, 170 $mM^{-1}.s^{-1}$) and (46 μm, 137 $mM^{-1}.s^{-1}$ respectively (Day et al., 2000). The substrate specificity of LPH for polyphenolic glycosides in admixture may be an interesting topic of further research.

The deglycosylation of polyphenolic glycosides results in the gradual increase of the aglycone so that it is free to interact with the bitter taste receptors (Roland et al., 2011) that trigger the enteroendocrine response that initiates satiety (Jang et al., 2007, Rozengurt, 2006). A better understanding of the deglycosylation of polyphenolic glycosides may affect the development of supplemental or nutraceautical products (Shahidi, 2009, Sun-Waterhouse et al., 2014, Dórea and da Costa, 2005, Sun-Waterhouse and Wadhwa, 2013). However, as discussed in the introduction of **chapter 8,** the rapid phase II and III metabolism of xenobiotics (such as polyphenolic aglycones) could mean that there is minimal activation of bitter taste receptors *in vivo.*

Purified polyphenolic compounds were also subjected to digestion in the presence of bile and/or pancreatin, and either active or heat treated BBMV preparation. In cases where polyphenolics were digested by biliopancreatic secretions alone no deglycosylation occurred further demonstrating the need for the BB phase in the digestion of polyphenolic glycosides. The presence of bile and/or pancreatin resulted in significant differences in the measured concentrations of the polyphenolics assayed. For future studies it may be beneficial to prepare digestate for LC-MS by the prior removal of protein, in case protein binding was an influential variable. In summary, the physiologically relevant BB *in vitro* digestion methodology that was developed for the hydrolysis of plant polyphenolic

compounds functioned well, and it was clear that polyphenolics that were glycosylated at the 4′ position were the preferred substrates.

9.5 Ways in which the experimental approach could be improved

There are a few things that in retrospect could have improved the quality of this work. Firstly, it would have been beneficial to have undertaken kinetic studies of BB enzyme activities in the presence of biliopancreatic secretions in order to further understand the inhibition of individual enzymes by those secretions. Secondly, in **Chapter 7** the kinetic activities of BBMV-bound, and solubilised BB enzymes were examined under several pH's. With 72 different assays to do in one day on the BBMV, soluble, amphipathic and aqueous fractions the second BBMV pellet (remaining following enzyme solubilisation) was not assayed. This omission does not detract greatly from the results, but its inclusion would have made the experimental work more robust.

Unfortunately, *in vitro* digestion including BB enzymes, does not cater for the absorption of nutrient monomers, bile salts, lipid oligomers and water during aboral transport through the small intestine. Digestive products and fluid that are absorbed rapidly *in vivo* via transcellular and paracellular pathways (Pappenheimer, 1993) will accumulate in the *in vitro* digestion method described here . Although during *in vitro* digestion some glucose monomers may be transferred into BBMV (Kessler et al., 1978) high concentrations of product could inhibit digestion (Semenza and Balthazar, 1974), however this is a common phenomena in most *in vitro* models.

9.6 The next step…

The adjunct BB digestive system developed for the digestion of plant polyphenolic compounds and whole foods has proved its capacity to hydrolyse polyphenolics.

However, as an *in vitro* system it needs to be tested against whole foods and/or their equivalents. Unfortunately under the constraints of this PhD there was not enough time to undertake these experiments. However, this would be the logical next step for this line of research. From there the system could be used as an adjunct to other models of biliopancreatic digestion, such as TIM-1 and DGM to help identify new bioactive derivatives of interest. The use of BB *in vitro* method could lead to the identification of dietary components that can initiate an enteroendocrine response that leads to satiety (bitter brake), could alter the amount or rate of glucose released from food *in vitro,* and could be used in the further identification of dietary allergens, such as peptide oligomers that cause allergic responses.

Another factor to consider is the benefit of using a consortium of enzymes rather than individual enzymes. Gastric lipase mediated digestion of lipids in an oil water emulsion may be impeded by the partitioning of proteins across the oil-water interface, and conversely may be augmented with the addition of gastric proteases (Lueamsaisuk et al., 2013). Similarly, incorporating BBMV may ensure that the terminal digestion of the main nutrients, CHO, protein and lipids, within a food matrix, is completed. Hence, this system would be beneficial for the *in vitro* digestion of whole foods that are assessed for starch digestibility (Alonso et al., 2000) or glycaemic response.

9.6.1 Using commercial enzymes to develop a standardised proxy BB digestion

One of the difficulties with using a consortium of enzymes, like those attached to BBMV, is the ability to standardise so that other researchers can achieve consistent results. A weakness in the BBMV *in vitro* method is that it cannot be accurately standardised, as seen in the difficulty in replicating the results in this thesis. If researchers prefer a system that can be standardised commercial enzymes may be used.

The use of commercially available BB enzymes equivalents means that researchers with little access to animal models may mimic BB digestion *in vitro*. However, there are caveats. Firstly, commercially prepared human BB enzymes are expensive; in order to be cost effective use of enzymes from different sources (other mammalian species, bacteria, and fungi) would be more practical, and more accessible. Secondly, the specificity, pH and temperature optimums of these enzymes can be significantly different to those human small intestinal enzymes and therefore should be rigorously tested prior to use. Thirdly with over one hundred BB hydrolases identified (McConnell et al., 2011) it would not be economically feasible to include the entire array of BB proxies into an *in vitro* system. Hence, an array of enzymes would need to be chosen from within the consortia of enzymes with sufficient specificity to represent BB digestion. Establishing a rationale and protocols for a standardisable proxy BB digestive phase was undertaken during the course of this PhD, and a body of work completed, as described in appendix 2.

9.7 Conclusion

By identifying aspects of *in vitro* digestion that can be modified to be more physiologically relevant, over time the process of *in vitro* digestion will be enhanced, bringing it closer in line with *in vivo* digestion. In that regard the aims of this thesis were fulfilled. Physiological and biochemical factors that may affect the function of *in vitro* digestions were identified, and suggestions on how to incorporate them into an *in vitro* digestion were proffered. Of those points, the most important one to *in vitro* digestion was the addition of a BB phase. A method for *in vitro* digestion was developed and the system was validated by the digestion of polyphenolic compounds.

In vivo digestion will never be able to be perfectly replicated *in vitro*. There are too many variables involved in the process because digestion involves the coordinated functioning of various organ systems regulated by neurological and hormonal input. However, it can

be made fit for purpose for defined applications with known limitations. The *in vitro* BB digestion phase puts us one step closer to having a physiologically relevant model of digestion.

10 Appendix 1

Table 49. Characteristics and kinetic information of intestinal brush border enzymes

Enzyme complex	EC number	Enzyme	Species	Estimated molecular weight kDa* (The UniProt Consortium, 2014)	Measured molecular weight† kDa	Form	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	V_{max} (μmol.min^{-1}.mg^{-1})	Author	Cofactor/ Induction/ Activation (The UniProt Consortium, 2014)
ALP	3.1.3.1	ALP	Porcine	58.03	(64x2) (Colbeau and Maroux, 1978)	BBMV-bound	10.5 (Fan et al., 1999)	10.5	*p* - nitrophenyl phosphate	0.66			4.27	(Fan et al., 2002)	Magnesium, Zinc
ALP	3.1.3.1	ALP	Porcine	58.03	(64x2) (Colbeau and Maroux, 1978)	Duodenal BBMV-bound	10.5	10.5	*p* - nitrophenyl phosphate	2.75			7.74	(Fan et al., 1999)	Magnesium, Zinc
ALP	3.1.3.1	ALP	Porcine	58.03	(64x2) (Colbeau and Maroux, 1978)	Jejunal BBMV-bound	10.5	10.5	*p* - nitrophenyl phosphate	0.64			4.31	(Fan et al., 1999)	Magnesium, Zinc
ALP	3.1.3.1	ALP	Porcine	58.03	(64x2) (Colbeau and Maroux, 1978)	Ileal BBMV-bound	10.5	10.5	*p* - nitrophenyl phosphate	2.71			3.53	(Fan et al., 1999)	Magnesium, Zinc
APN	3.4.11.2	APN	Human	109.54	(160,140) (Hauri et al., 1985)	Triton X 100 purified enzyme	7.5	.	Leu-naphtyl-amide	52			59.5	(Caporale and Troncone, 1988)	Zinc
APN	3.4.11.2	APN	Porcine	108.83	(162) (Sjöström et al., 1978)	BBMV-bound	7 – 7.5 (Feracci et al., 1981)	7	L-alanine-p-nitroalanine chloride	2.33			9.55	(Fan et al., 2002)	Zinc
APN	3.4.11.2	APN	Porcine	108.83	(162) (Sjöström et al., 1978)	Emulphogen/ pepsin solubilized purified	7 – 7.5	7	L-alanine-p-nitroalanine chloride	0.87	222	$2.55E^5$		(Feracci et al., 1981)	Zinc
APA	3.4.11.7	APA	Porcine	108.28	247 (Benajiba and Maroux, 1980)	Emulphogen/ pepsin solubilized purified	7 – 7.5	7	Glutamic acid α – *p* nitroanilide	0.98	208	$2.12E^5$		(Feracci et al., 1981)	Zinc
ACE	3.2.1.-/ 3.4.15.1	ACE	Human	149.72	184 (Hauri et al., 1985)	Cloned purified	.	7.4	Angiotensin I	0.019	3.5	$1.8E^5$		(Rice et al., 2004)	Zinc, chloride

Enzyme complex	EC number	Enzyme	Species	Estimated molecular weight kDa* (The UniProt Consortium, 2014)	Measured molecular weight† kDa	Form	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	V_{max} (μmol. min^{-1}. mg^{-1})	Author	Cofactor/ Induction/ Activation (The UniProt Consortium, 2014)
ACE	3.2.1.-/ 3.4.15.1	ACE	Rat	150.91	160 (Erickson et al., 1992)	Triton X114 solubilized purified	8.3-8.5 (Yoshioka et al., 1987)	8.3	Bz -Gly-His-Leu	1.9ǂ	303	$1.6E^5$		(Erickson et al., 1992)	Zinc, chloride
ACE	3.2.1.-/ 3.4.15.1	ACE	Rat	150.91	160 (Erickson et al., 1992)	Triton X100 solubilized purified	8.3-8.5 (Yoshioka et al., 1987)	7	Bz -Gly-Ala-Pro	0.73‡	719	$9.8E^5$	254	(Erickson et al., 1992)	Zinc, chloride
ACE	3.2.1.-/ 3.4.15.1	ACE	Rat	150.91	160 (Erickson et al., 1992)	BBMV-bound	8.3-8.5 (Yoshioka et al., 1987)	7	Bz -Gly-Ala-Pro	0.7			1.05	(Yoshioka et al., 1987)	Zinc, chloride
ACE	3.2.1.-/ 3.4.15.1	ACE	Rat	150.91	160 (Erickson et al., 1992)	BBMV-bound	8.3-8.5 (Yoshioka et al., 1987)	8.5	Bz -Gly-His-Leu	1			0.261	(Yoshioka et al., 1987)	Zinc, chloride
ACE 2	3.4.17.23	ACE 2	Human	92.46		Cloned purified	.	8.5	Angiotensin I	0.087	2.9	$3.3E^4$		(Rice et al., 2004)	Zinc, chloride
DPPIV	3.4.14.5	DPPIV	Porcine	88.24	230 (137x2) (Svensson et al., 1978)	Triton X100 solubilized purified	7.4–8 (Lojda, 1979)	8	Glycyl-L-proline nitroanilide	0.24				(Svensson et al., 1978)	.
ENT	3.4.21.9	ENT	Bovine	114.77	145 (57,82) (Anderson et al., 1977)	Triton X100 solubilized purified	7.8 (Maroux et al., 1971)	8	Trypsinogen	0.24	11.7	$4.86^{\wedge-2}$		(Mikhailova and Rumsh, 2000)	.
GP2	3.4.17.21	GP2	Porcine	84.52	120	Papain solubilized purified	6.5	6.5	Folyl-g -Glu-g-[14C]Glu	$3.9^{\wedge-3}$			0.338	(Halsted et al., 1998)	Zinc
LPH	3.2.1.108 / 3.2.1.62	Lactase	Sheep	.	.	Papain solubilized purified	4.5–6	5	Lactose	8.8			14.5	(Rivera-Sagredo et al., 1992)	.
LPH	3.2.1.108 / 3.2.1.62	Phlorizin	Sheep	.	.	Papain solubilized purified	4.5–6	5.9	Phlorizin	$2.^{\wedge-3}$			0.22	(Rivera-Sagredo et al., 1992)	.

Enzyme complex	EC number	Enzyme	Species	Estimated molecular weight kDa* (The UniProt Consortium, 2014)	Measured molecular weight† kDa	Form	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	V_{max} (μmol. min^{-1}. mg^{-1})	Author	Cofactor/ Induction/ Activation (The UniProt Consortium, 2014)
LPH	3.2.1.108 / 3.2.1.62	LPH	Human	218.59	160 (Naim et al., 1987, Skovbjerg et al., 1984)	Triton X100 solubilized purified	5.8 - 6	6	Lactose	21				(Skovbjerg et al., 1981)	.
LPH	3.2.1.108 / 3.2.1.62	LPH	Human	218.59	160 (Naim et al., 1987, Skovbjerg et al., 1984)	Triton X100 solubilized purified	5.8 - 6	6	Phlorizin	0.44				(Skovbjerg et al., 1981)	.
LPH	3.2.1.108 / 3.2.1.62	Lactase	Rat	217.27	(130) (Büller et al., 1987)	Papain solubilized purified	5.5 – 5.8	5.5	Lactose	25				(Asp and Dahlqvist, 1968)	.
LPH	3.2.1.108 / 3.2.1.62	LPH	Rat	217.27	(130) (Büller et al., 1987)	Triton X100 solubilized purified	5.5 – 5.8	6	Lactose	16	47	2.9^3	5	(Mackey et al., 2002)	.
MGAM	3.2.1.20/ 3.2.1.3	MGAM	Porcine	.	330 (135,125)	Papain/Triton X100 solubilized purified	6 – 7	6	Maltose	1.74				(Sorensen et al., 1982)	.
MGAM	3.2.1.20/ 3.2.1.3	Gluco - amylase	Human	209.85	312 (Kelly and Alpers, 1973)	Cloned purified	6 (Auricchio et al., 1965)	7	Maltose	5.53	22	$7.7E^3$		(Ren et al., 2011)	.
MGAM	3.2.1.20/ 3.2.1.3	Maltase	Human	209.85	335 (Naim et al., 1988b, Hauri et al., 1985)	Cloned purified	6 (Auricchio et al., 1965)	4.8	Maltose	6.17	47.8	$4.1E^3$		(Ren et al., 2011)	.
MGAM	3.2.1.20/ 3.2.1.3	Maltase	Human	209.85	335 (Naim et al., 1988b, Hauri et al., 1985)	Cloned purified	6 (Auricchio et al., 1965)	6.5	Isomaltose	227	13	57		(Sim et al., 2010)	
MGAM	3.2.1.20/ 3.2.1.3	Maltase	Human	209.85	335 (Naim et al., 1988b, Hauri et al., 1985)	Cloned purified	6 (Auricchio et al., 1965)	6.5	Maltose	4.3	111	$2.6E^4$		(Sim et al., 2010)	.

Enzyme complex	EC number	Enzyme	Species	Estimated molecular weight kDa* (The UniProt Consortium, 2014)	Measured molecular weight† kDa	Form	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	V_{max} (μmol. min^{-1}. mg^{-1})	Author	Cofactor/ Induction/ Activation (The UniProt Consortium, 2014)
NEP	3.4.24.11	Neprilysin	Human	85.51	150 (Guan et al., 1988)	Cloned purified	.	7.4	Angiotensin I	0.055	34.1	$6.2E^5$		(Rice et al., 2004)	Zinc
NC	3.5.1.23	NC	Human	85.82	116	Solubilized purified	7.5	7	Octanoyl-sphingosine	0.013			0.807	(Olsson et al., 2004)	+ve: Na^+, $Ca2^+$, Mg^{2+},Mn^{2+}
NC	3.5.1.23	NC	Rat	83.49	116	Bile solubilized purified	6	7	Octanoyl-sphingosine	0.071			160	(Olsson et al., 2004)	.
LPB1	3.1.1.4 / 3.1.1.5	PLB1	Rat	161.09	130	Papain solubilized purified	8 – 9 (Tojo et al., 1998)		Phosphatidyl-choline	0.011			13.9	(Rigtrup et al., 1994)	+ve bile salts (Rigtrup et al., 1994)
SI	3.2.1.48/ 3.2.1.10	Sucrase	Porcine	.	265 (170,140) (Danielsen, 1992)	BBMV-bound		6.1	Sucrose	16.57			1.3	(Fan et al., 2002)	.
SI	3.2.1.48/ 3.2.1.10	SI	Rat	210.35	320 (Cezard et al., 1979)	Autolyzed solubilized purified	5.9 (50mM Na^+) (Kolínská and Kraml, 1972)	6.1	Isomaltose	5	74	$1.48E^4$		(Gray et al., 1979)	+ve sodium (Kolínská and Kraml, 1972)
SI	3.2.1.48/ 3.2.1.10	Isomaltase	Human	209.45	245 (130,145)	Cloned purified	5.9 (50mM Na^+) (Kolínská and Kraml, 1972)	6.5	Isomaltose	11.1	97	$8.8E^3$		(Sim et al., 2010)	.
SI	3.2.1.48/ 3.2.1.10	SI	Rat	210.35	320 (Cezard et al., 1979)	Autolyzed solubilized purified	5.9 (50mM Na^+) (Kolínská and Kraml, 1972)	6.1	Sucrose	19	120	$6.3E^3$		(Gray et al., 1979)	+ve sodium (Kolínská and Kraml, 1972)

Enzyme complex	EC number	Enzyme	Species	Estimated molecular weight kDa* (The UniProt Consortium, 2014)	Measured molecular weight† kDa	Form	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	V_{max} (μmol. min^{-1}. mg^{-1})	Author	Cofactor/ Induction/ Activation (The UniProt Consortium, 2014)
SI	3.2.1.48/ 3.2.1.10	Sucrase	Rat	210.35	320 (Cezard et al., 1979)	Autolyzed solubilized purified	5.9 (50mM Na^+) (Kolínská and Kraml, 1972)	6.1	Sucrose	19	15	$7.9E^2$		(Gray et al., 1979)	+ve sodium (Kolínská and Kraml, 1972)
SI	3.2.1.48/ 3.2.1.10	Isomaltase	Rat	210.35	320 (Cezard et al., 1979)	Autolyzed solubilized purified	5.9 (50mM Na^+) (Kolínská and Kraml, 1972)	6.1		5	30	$6E^3$		(Gray et al., 1979)	+ve sodium (Kolínská and Kraml, 1972)
Trehalase	2.2.1.28	Trehalase	Rat	63.49	65.5	Triton X100 solubilized /purified	5.5 – 5.7	6.8	Trehalose	10				(Riby and Galand, 1985)	.
Trehalase	2.2.1.28	Trehalase	Rabbit	65.52	75	Emulphogen solubilized /purified	5.5 - 6	6	Trehalose	3.5				(Galand, 1984)	.
Trehalase	2.2.1.28	Trehalase	Rat	63.49	65.5 (Riby and Galand, 1985)	Triton X100 solubilized purified	5.5 – 5.7	5.7	Trehalose	5.4				(Sasajima et al., 1975)	.

Table 50. Characteristics and kinetic information of key gastric and pancreatic enzymes.

Enzyme	EC number	Species	Estimated molecular weight (kDa)* (Consortium 2014)	Measured molecular weight † (kDa)	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	V_{max} (μmol.min^{-1}.mg^{-1})	Author	Cofactor/ induction/ activation (Consortium 2014)
Pepsin	3.4.23.1	Porcine	41.26	34.64 (Tang et al., 1973)	1.5-2 (Piper and Fenton, 1965)	4	Z-His-Phe(NO2)-Phe-Ala-Ala-OMe	0.13	28	2.2^3		(Voynick and Fruton, 1971)	Acidic pH for activation
Pepsin	3.4.23.1	Human	42	34.1 (Mills and Tang, 1967)	1.7-3.4	2	N-acetyl-L-phenylalanyl –L 3,5-diodotyrosine	0.117‡			0.06	(Becker and Rapp, 1979)	Acidic pH for activation
Pepsin	3.4.23.1	Rat	.	35.5	2.1–3.1	.	AcPhe-Tyr(I_2)	1E^{-4}	0.011	105		(Furihata et al., 1980)	Acidic pH for activation
Gastric lipase	3.1.1.3	Rabbit	40.4 (Moreau et al., 1988a)	49 (Moreau et al., 1988a)	4-6 (Moreau et al., 1988a)	4	Medium chain triacylglycerol (0.19um)	6.2¶			17.9§	(Borel et al., 1994)	.
Gastric lipase	3.1.1.3	Pig	.	.	7 (Moreau et al., 1988b)	6.5	tributryin	0.62			45.7‖	(Jensen et al., 1997)	.
Gastric lipase	3.1.1.3	Human	45.24	44	4 – 6	5.3	tributryin	21				(Tiruppathi and Balasubramania, 1982)	.
Trypsin	3.4.21.4	Human	26.56	21	7.8	8	α- *N*-benzoyl-L-arginine ethyl ester	0.012	58		4.9E^{6}	(Anderson et al., 1981)	Calcium Enteropeptidase activates trypsinogen → zymogen activation (Light and Janska, 1989)

Enzyme	EC number	Species	Estimated molecular weight (kDa)* (Consortium 2014)	Measured molecular weight † (kDa)	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	V_{max} ($\mu mol.min^{-1}.mg^{-1}$)	Author	Cofactor/ induction/ activation (Consortium 2014)
Trypsin	3.4.21.4	Canine	26.17	.	7-9	.	Bz- D- L-arginine *p* -nitroanilide	1.1^-6				(Ohlsson and Tegner, 1973)	Calcium Enteropeptidase activates trypsinogen → zymogen activation (Light and Janska, 1989)
Trypsin	3.4.21.4	Porcine	24.41	23.43 (Dallas Johnson et al., 2002)	8.5 (Dallas Johnson et al., 2002)	8.1	Bz- D- L-arginine *p* -nitroanilide	0.91	2.72	3^3		(Outzen et al., 1996)	Calcium Enteropeptidase activates trypsinogen → zymogen activation (Light and Janska, 1989)
Chymotrypsin	3.4.21.1	Bovine	25.67	.	6.5-7 (Schwert and Takenaka, 1955)	7.8	Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide	0.043	45	1^6		(DelMar et al., 1979)	.
Chymotrypsin	3.4.21.1	Bovine	25.67	.	6.5-7 (Schwert and Takenaka, 1955)	7.5	Ac-Ala-Ala – Pro-Phe-*p*-nitroanilide	2.3^-7	9.8	4.3^4		(Zimmerman and Ashe, 1977)	.
Elastase	3.4.21.36	Porcine	28.82	25	7.5–9 (Bieger and Scheele, 1980)	7.5	Ac-Ala-Ala – Pro-Ala-*p*-nitroanilide	9.5^-7	43	4.5^4		(Zimmerman and Ashe, 1977)	Calcium
Elastase	3.4.21.36	Rat	28.89	25 (Bieth et al., 1989)	.	8	Succinoyl-Ala-Ala-Pro-Ala- *p*-nitroanilide	0.18	4.89	2.7^4		(Largman, 1983)	Calcium

Enzyme	EC number	Species	Estimated molecular weight (kDa)* (Consortium 2014)	Measured molecular weight † (kDa)	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	V_{max} ($\mu mol.min^{-1}.mg^{-1}$)	Author	Cofactor/ induction/ activation (Consortium 2014)
Elastase	3.4.21.36	Human	27.8	29.3 (Largman et al., 1976)	.	8	Succinoyl-Ala-Ala-Pro-Ala- *p*-nitroanilide	2.4	0.34	142		(Largman, 1983)	
CPA	3.4.17.1	Human	47.14	35	7.5–8	7.5	Bz-Gly-Gly-O-Phe	20	7.7^-4	3.8^-8		(Peterson et al., 1976)	Zinc
CPA	3.4.17.1	Bovine	47.08	34.6 (Bargetzi et al., 1963)	7.5 (Ebata and Miyazaki, 1967)	7.5	Bz-Gly-Gly-O-Phe	0.3	500	1.7^6		(Spilburg et al., 1977)	Zinc
CPB	3.4.17.2	Human	47.37	34.25	7	7.4	Hippuryl-arginine	0.277			555	(Marinkovic et al., 1977)	Zinc
CPB	3.4.17.2	Human	47.37	27.7	7 (Marinkovic et al., 1977)	7.65	Hippuryl-arginine	0.65				(Geokas et al., 1975)	Zinc
Pancreatic lipase	3.1.1.3	Human	51.16	48 (De Caro et al., 1977)	4-5 (Interfacial binding) (Ranaldi et al., 2008);7.5 (tributryin/ with colipase) (Vandermeers et al., 1974)	9.2	Olein	15.6			$2.25E^4$	(Lowe, 1992)	Co-lipase in the presence of bile salts
Pancreatic lipase	3.1.1.3	Porcine	50.08	55	8.9 (Bagi et al., 1997)	8.9	Trioctanoin			8.46^5		(Lagocki et al., 1973)	Co-lipase in the presence of bile salts

Enzyme	EC number	Species	Estimated molecular weight (kDa)* (Consortium 2014)	Measured molecular weight † (kDa)	Optimal pH	Assay pH	Assay substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	V_{max} (μmol.min^{-1}.mg^{-1})	Author	Cofactor/ induction/ activation (Consortium 2014)
Bile salt activated lipase (BSAL)	3.1.1.13/ 3.1.1.3	Rat	67.04	65-70 (Iijima et al., 1998)	~7-8 (Chahinian et al., 2010)	7	Triacetin	0.015		28	0.323	(Hui et al., 1993)	Galectin 10, bile salts with 7-OH group
BSAL	3.1.1.13/ 3.1.1.3	Bovine	65.16	60	7-8	7	Tributyrin	1.5	215	1.4^5		(Chahinian et al., 2010)	Galectin 10, bile salts with 7-OH group
PLA2	3.1.1.4	Ovine	13.817	14	8.7	8	L-α-dioctanoyl-lecithin	3			1300#	(Dutilh et al., 1975)	Calcium
PLA2	3.1.1.4	Porcine	16.28	14 (Beiboer et al., 1995)	9–9.5 (Van Oort et al., 1985) (Van Oort et al., 1985)	8	Dipalmitoyl-phophatidyl-choline	2	55	2.8^4		(Menashe et al., 1986)	Calcium
PLA2	3.1.1.4	Porcine	16.28	14	9–9.5 (Van Oort et al., 1985)	8	rac -1,2-dihexamoyldi -thiolecithin	0.85	0.7	824		(Beiboer et al., 1995)	Calcium
***α*-amylase**	3.2.1.1	Porcine	57.09	50 (25x2) (Robyt et al., 1971)	6.9 (Wakim et al., 1969)	6.9	maltapentaose	1.08	1360			(Prodanov et al., 1984)	Calcium, chloride
***α*-amylase**	3.2.1.1	Human	57.71	54 (Stiefel and Keller, 1973)	7 (Matsuura et al., 1983)	7	maltapentaose	2.3				(Narimasa et al., 1979)	Calcium, chloride

***** Estimated molecular weight based on protein sequence; † Molecular weight based on experimental evidence. Where available the molecular weight of subunits are given in parentheses; ‡ mmol; ¶ mg/ml; § μmol of FFA/min; ‖ μmol/min/gram tissue; #μequiv. Acid released.min^{-1}.mg^{-}

11 Appendix 2

11.1 Replicating the brush border phase of small intestinal digestion *in vitro*

Enzymes of the small intestinal brush border complete the digestion of nutrient oligomers, which bypass previous digestion. Currently, there are no tested *in vitro* methodologies that simulate brush border digestion. We describe an economical formulation of brush border enzyme equivalents that would be suitable for use as a subsidiary to current *in vitro* systems that are designed to replicate the human digestive process. In conjunction with this we have developed rationales for the selection of particular enzymes from the array of brush border enzymes that are produced and for ambient conditions that represent those in the peripheral peri–apical space of the small intestinal lumen. This process has been undertaken with due regard to the biochemical dynamics and requirements of the component enzymes.

11.1.1 Introduction

Enzymes from the mammalian small intestinal brush border (BB) have long been recognised as playing an important role in food digestion (Miller and Crane, 1961a, Holmes and Lobley, 1989). Broadly speaking BB digestive enzymes hydrolyse nutrient oligomers that remaining following the hydrolytic action of buccal, gastric and pancreatic enzymes (Beck, 1973, Kenny et al., 1983). Their substrates include nutrients that are unable to be hydrolysed by pancreatic enzymes (*e.g.* disaccharides) (Skovbjerg et al., 1981, Rodriguez et al., 1984), bioactive nutrients (*e.g.* sphingolipids) (Vesper et al., 1999), and allelopathic chemicals (*e.g.* plant polyphenolics) that are modified prior to their absorption and detoxification (Day et al., 2000, Nemeth et al., 2003b, Sesink et al., 2003). The importance of this process is exemplified by disorders characterised by

permanent or temporary deficiencies in BB enzymes. Sucrase-isomaltase (SI, EC 3.2.1.48/3.2.1.10) and lactase-phlorizin hydrolase (LPH, EC 3.2.1.108/3.2.1.62) deficiencies lead to abdominal discomfort and osmotic diarrhoea (Bayless and Christopher, 1969), and enteropeptidase deficiency (EC 3.4.21.9) leads to gross protein malabsorption, diarrhoea, oedema and failure to thrive (Holzinger et al., 2002).

In spite of their importance none of the current *in vitro* methodologies, that have been developed to standardise the digestive process (Wickham and Faulks, 2012, Minekus and Havenaar, 1996, Kong and Singh, 2010, Tharakan et al., 2010, Hur et al., 2011, *Hollebeeck et al., 2013),* incorporate BB enzymes. This is likely due to their diversity, and the chemical and physical complexities of BB enzyme secretion and action (Hooton et al., 2015). A study of the murine brush border membrane vesicle (BBMV) proteome identified 103 BB hydrolase enzymes, which include a range of oligosaccharidases, oligopeptidases and lipolytic enzymes (McConnell et al., 2011). The substrate specificities of many of these enzymes are distinct from enzymes secreted by gastric and pancreatic glands (Hooton et al., 2015) and so *in vitro* digestion by buccal gastric and pancreatic enzymes alone may not be representative of *in vivo* digestion.

The physiological complexities of BB enzyme production and secretion complicate their biochemical dynamics. Rather than being localised to the apical membrane of microvilli (Ugolev, 1965) BB enzymes are shed, along with the apical membrane to which they are attached, into the adjacent peri-apical space (McConnell and Tyska, 2007, McConnell et al., 2009). Brush border digestive enzymes that are initially adhered to a BBMV, following vesiculation, may be released by the action of biliopancreatic secretions (Semenza, 1986, Kenny et al., 1983, Maestracci, 1976) or shear force (Maestracci, 1976). While BBMV have the capacity to transit to the central lumen (McConnell et al., 2009, Halbhuber et al., 1994) their rate of diffusion through the peripheral unstirred water layer

is likely to be slow as a result of their large size (mean diameter 100 ηm) (Boffelli et al., 1997, McConnell et al., 2009, Perevucnik et al., 1985) compared with that of solubilised BB enzymes (70 - 320 kDa) (Holmes and Lobley, 1989). These processes are not quantifiable, but may have important consequences regarding the transit of enzymes into the lumen, and to the microclimate in which they operate.

The task of emulating BB digestion *in vitro* is also complicated by the lack of detailed knowledge regarding the physicochemical environment in which BB enzymes operate (Hooton et al., 2015). The conditions in which BB enzymes occur *in vivo* varies depending on location, *i.e*. surrounding a BBMV (Wistrand and Kinne, 1977, Murer et al., 1976), overlying the epithelia (Lucas et al., 1975, Lucas, 1983), along the longitudinal axis of the villus (Daniel et al., 1989) or in luminal contents (Evans et al., 1988). All of which vary with anatomical location (Lucas, 1983). Choosing a physiologically relevant pH that is in keeping with the optimal pH of proxy BB enzymes is therefore important in the establishment of a functional BB phase.

In this paper we describe the development of a simplified economical formulation of BB digestive enzyme equivalents that emulate the BB digestion of nutrient oligomers, and would be suitable for use in an *in vitro* system designed to replicate the human digestive process. We describe rationales for the selection of proxy BB enzymes, from the many that are produced commercially, and for the selection of particular ambient conditions that approach those reported in the peripheral space of the small intestinal lumen. This is achieved with due regard to the biochemical dynamics and requirements of the component enzymes.

11.1.2 Rationale for the choice and use of enzymes

As it is not practicable to include the entire array of BB enzymes into an *in vitro* system a rationale was developed to justify the incorporation of particular enzymes. We

endeavoured to acquire intestinal BB enzymes, but they were principally recombinant, which made the price prohibitive for their use in the routine testing of food digestibility. Similarly, it was impractical to devise a system that would assess the extent of degradation of the entire spectrum of nutrients and plant allelochemicals that are found in the human diet. Hence, we considered only those BB enzymes that contribute to the digestion of the three main classes of macronutrients: protein, carbohydrates and fats. Further, we limited the range of such enzymes to those whose affiliated dietary components produce molecular fragments in significant quantities to warrant their addition, *i.e.* ≥ 2 % of dietary energy. The latter was particularly important with regard to the omission of BB enzymes that specifically digest rarer lipids. For example, alkaline sphingomyelinase (AS, EC 3.1.4.12) and neutral ceramidase (NC, EC 3.5.1.23) are important in the hydrolysis of sphingolipids (Vesper et al., 1999). The hydrolysis of which produces bioactive compounds that effect the regulation of important cellular processes (Vesper et al., 1999, Merrill et al., 1997, Merrill Jr et al., 1997). Together AS and NC comprise 64 % of spectra identified as lipolytic enzymes in the murine BBMV proteome (McConnell et al., 2011). While sphingolipids are found in a wide range of foods in the western diet the average intake is low at approximately 0.3–0.4 g per day (Vesper et al., 1999). This is equivalent to ~ 0.1–0.4 % of daily energy intake (University of Otago and Ministry of Health, 2011). This does not meet the required ≥ 2 % threshold of dietary energy and as such sphingolipid hydrolysing enzymes were not included in the array.

Brush border enzymes with substrates in the three classes of macronutrients were also limited to those that could cleave substrates at molecular sites that were distinct from those cleaved by enzymes of gastric and pancreatic origin. For example, pancreatic secretions contain phospholipase enzymes such as pancreatic phospholipase A2 (PLA2) (EC 3.1.1.4) and pancreatic lipase related protein 2 (EC 3.1.1.26/3.1.1.3). Whereas BB

phospholipases include phospholipase A2 group IVC (PLA2-G4C, EC 3.1.1.4) and phospholipase B1 (PLB1, EC 3.1.1.4/3.1.1.5). (McConnell et al., 2011). In this instance the EC 3.1.1.4 activity of BB PLA2-G4C and PLB1 is made redundant by presence of pancreatic PLA2. Further, the lysophospholipase and non-positional triacylglycerol lipase activity (Semenza, 1986) of PLB1, including the ability to hydrolyse lipids and phospholipids(Gassama-Diagne et al., 1989) at the SN_2 position (IUBMB, 2013), is represented in pancreatic secretions by bile salt activated lipase (EC 3.1.1.13/3.1.1.3) .

It is important not to abbreviate the suite of enzymes to those which act only on oligomers derived from a particular macronutrient. This is significant because recent work indicates that enzymes specific for other macronutrients may act synergistically in the digestion of a given macronutrient. For example, the gastric lipase (EC 3.1.1.3) digestion of lipids in an oil water emulsion may be impeded by the partitioning of proteins across the oil-water interface (Lueamsaisuk et al., 2013). Correspondingly the enzymatic activity of gastric lipase may be augmented by the addition of proteolytic enzymes that hydrolyse peptides from these proteins allowing them to diffuse away (Lueamsaisuk et al., 2013). We therefore consider that in addition to a full array of gastric and pancreatic enzymes a consortium of BB enzyme equivalents, that are capable of digesting oligomers derived from both carbohydrate and protein, are used regardless of the substrate being studied.

Where alternatives to particular BB enzymes were used (*e.g*. those from fungal sources) they were chosen on the basis that their activity demonstrated value for money (*i.e*. high Units of activity per NZD), and that they were likely to function in ambient conditions similar to those of human BB enzymes.

11.1.3 Replication of ambient operating conditions

The ambient pH in which the chosen enzymes will operate was based on those reported in the peri-apical region of the enterocyte where the bulk of extracellular BB enzymes are

thought to operate, *i.e*. at or close to the apical membrane of the small intestinal mucosa. The pH in the peri–apical space of the proximal jejunum and distal ileum is 6.1 and 7.3 respectively .

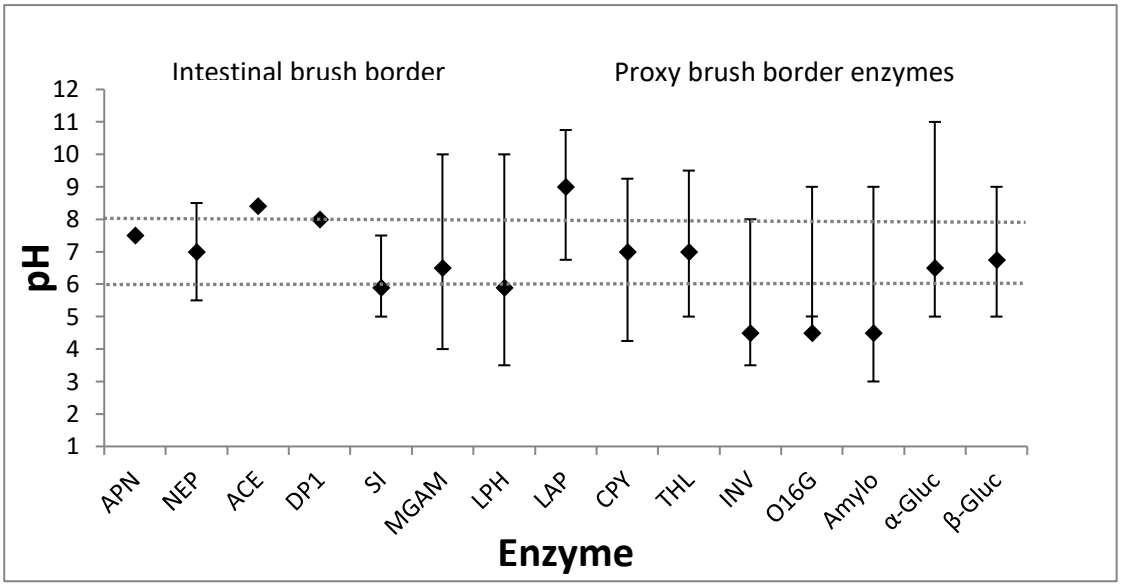

Figure 34. The functional pH range of brush border and proxy brush border enzymes. Diamonds indicate the pH optimum. The bars indicate the functional pH range of BB and proxy BB enzymes. The functional pH range of THL, INV, O16G, Amylo, α-Gluc and β-Gluc was supplied by the manufacturer.

The enzymes chosen to represent the oligosaccharidase and oligopeptidase consortium displayed two broad pH ranges clustered around 6 and 8 respectively (see figure 34). These pH concentrations were therefore chosen for forthcoming *in vitro* analysis in order to ensure the enzymatic efficiency of incorporated enzymes, while still being in keeping with the pH optimum of intestinal BB enzymes.

To ensure that the chosen proxy BB enzymes functioned efficiently divalent cations were added to the buffering solution. This was important as many of the proxy BB enzymes require cofactors. Leucine aminopeptidase (LAP) (EC 3.4.11.2) requires zinc (Himmelhoch, 1969), magnesium (Lin and Van Wart, 1982, The UniProt Consortium, 2014) and manganese for activity (The UniProt Consortium, 2014); zinc and calcium are

required for thermolysin (EC 3.4.24.27) activity (The UniProt Consortium, 2014). The concentration of these cations was determined based on the distribution of these elements within cells, and were included at levels below those that are deemed toxic (Williams and Fraústo da Silva, 2000). Further, as commercial supplies of enzymes are likely to contain these elements the concentration of each cation added was based on the low end of the advised concentration, table 51. Phosphate buffer was chosen as the medium for enzymatic reactions as it allowed the use of one buffer for pH 6 and 8 (10mM phosphate buffer, 50mM mannitol). Divalent cations have a propensity to form complexes with phosphate and precipitate from solution so we monitored buffer stocks and incubations for precipitates. The pH 6 buffer did not form precipitates, but the pH 8 buffer did if the buffer was autoclaved with cations. Hence, the phosphate buffer was autoclaved and the cations were added afterwards following filtering through a 0.22 µm filter.

Table 51. Concentration of important intracellular divalent cations added to buffer

Ionic species	Concentration (mM)
Zinc	$1.00x10^{-08}$
Magnesium	1
Manganese	0.001
Calcium	$1.00x10^{-04}$

11.1.4 The effect of biliopancreatic secretions on proxy enzyme activity

As BB enzymes are exposed to pancreatic proteases *in vivo* it was important to consider whether the chosen enzymes were susceptible to proteolysis. Since proteins that have undergone post-translational modification have greater stability (Mann and Jensen, 2003) we endeavoured to source enzymes that had undergone normal processing and expression. In particular we wanted to choose enzymes that were extensively

glycosylated. Glycosylation increases resistance to proteolysis (Kingsley et al., 1986, Vaňková et al., 1994) and the majority of BB enzymes tend to be highly glycosylated (Naim et al., 1988b, Kelly and Alpers, 1973, Naim et al., 1988a). Many of the commercially available enzymes are recombinant so they do not have the compliment of glycosidic residues that normal expression would engender.

Given that bile must transit the peri-apical space to be absorbed, it is evident that BB enzymes will be exposed to its action. The hydrophobic components of soluble enzymes could more vulnerable to the action of bile so it was necessary to verify that their activity was unaffected by bile at the chosen pH. This was determined experimentally.

11.1.5 The relative proportions of the enzymes

Quantitative difficulties and kinetic differences effectively precluded the use of BB enzymes in proportions that were based on the physiological rates of secretion. The relative proportions of BB enzymes vary physiologically (McConnell et al., 2011), and the specific activities of individual enzymes vary with factors such as the anatomical location where the small intestine is sampled (Ferraris et al., 1992, Fan et al., 1999), diet (Xiao et al., 2011), time of harvest (Saito et al., 1976) and age (Fan et al., 2002). Further, the specific activity of BB enzymes are reported to differ between soluble and BBMV-bound forms (Kozak and Tate, 1982, Fan et al., 2002), with biochemical methodology (Galand and Forstner, 1974b), pH, chemical milieu (Kolínská and Kraml, 1972, Vasseur et al., 1982) and substrate (Kozak and Tate, 1982). We combined the chosen proxy enzymes in proportions that reflected their reported proportions in proteomic data (McConnell et al., 2011). The proportions were derived from spectral counts and are technically not a direct reflection of enzyme proportions as the identification of some peptide fragments is more difficult, and larger proteins will yield more peptide fragments (thus more spectra). However, no other tangible data is available so the concentrations of

each enzyme were chosen based on these proportions and on preliminary enzyme characterization data.

11.1.6 The choice of enzymes

Consortiums of gastric and pancreatic enzymes act in concert to hydrolyse dietary nutrients in gastric and small intestinal chyme. Brush border enzymes continue this hydrolysis with enzymes whose substrate specificity is complimentary to the action of pancreatic enzymes. For example pancreatic α-amylase (EC 3.1.1.1) is a carbohydrase endoenzyme that is unable to hydrolyse terminal glucose residues or α-1,6 linked glucosidic residues (Beck, 1973). The BB oligosaccharidases SI and maltase-glucoamylase (MGAM, EC 3.2.1.20/3.2.1.3) then act synergistically to hydrolyse those residues that PA cannot hydrolyse (Van Beers et al., 1995a). It follows then that the chosen suite of proxy BB enzymes should contain enzymes with sufficient range of specificity to convert the bulk of food polymers to their component monomers. In terms of the complex array of BB enzymes the task reduces to that of ensuring the linkage specificities complement those of pancreatic and gastric enzymes.

11.1.6.1 The peptidase consortium

The range of oligopeptides that are produced by the action of gastric and pancreatic enzymes will be biased according to the amino acid content of the substrate and the relative proportions of pancreatic enzymes present in the lumen. The action of pancreatic proteolytic enzymes is limited in two respects. Firstly, the endopeptidases all have the same mode of action, *i.e.* they all belong to the serine S1 protease family that have a serine residue contributing to the catalytic triad at the active site (Hedstrom, 2002). Secondly, while *C*-terminal exopeptidases are present in pancreatic secretions there are no *N*-terminal exopeptidases (Beck, 1973). Brush border oligopeptidases include a suite of

enzymes are categorised into four distinct groups based on their specificity, *i.e.* *N*-terminal exopeptidases, *C*-terminal exopeptidases, endopeptidases and dipeptidases. The aim was for one enzyme from each group to be chosen to represent BB oligopeptidases.

11.1.6.1.1 N-terminal exopeptidase

Taking into account the substrate specificities of the principal *N*-terminal exopeptidases (**chapter 2**) APN was chosen as the *N*-terminal exopeptidase to represent this group. Firstly, APN is the most prevalent oligopeptidase (McConnell et al., 2011), and secondly, although the primary physiological role of APN is in the cleavage of basic amino acid residues (Benajiba and Maroux, 1980) it is capable of hydrolysing a wide range of substrates (Wong et al., 2012), including charged residues (Benajiba and Maroux, 1980) and proline (The UniProt Consortium, 2014). However, when the penultimate amino acid is proline, a dipeptide is released (The UniProt Consortium, 2014). In addition to its role as an N-terminal peptidase APN is also able to hydrolyse dipeptides (Kozak and Tate, 1982). This activity augments the activity of the chosen dipeptidase.

An economical source of APN was not found. Instead porcine renal LAP (Sigma-Aldrich Ltd) was procured. Leucine aminopeptidase is a cytosolic aminopeptidase from the peptidase M17 family that requires zinc ions for activity (Himmelhoch, 1969, Rawlings et al., 2012). It is an enzyme with broad specificity (Hill and Smith, 1957) that prefers *N*-terminal leucine, but hydrolyses most *N*-terminal amino acids from polypeptides (Hill and Smith, 1957) and dipeptides (Smith et al., 1952). The optimal pH of LAP is 9 (Lin and Van Wart, 1982, Van Wart and Lin, 1981), which is somewhat higher than intestinal APN which has a pH optima of 7.5 (Caporale and Troncone, 1988).

It is difficult to compare the kinetic activity of renal LAP with that of intestinal APN owing to variations in pH, enzyme form and methodological conditions. The K_m for the porcine LAP hydrolysis of leucine *p*-nitroanilide at pH 9 (10 % v/v dimethyl sulfoxide)

is 0.028 mM, the k_{cat} 0.5 s^{-1} and the k_{cat}/K_m 0.18 $M^{-1}.s^{-1}$ (Lin and Van Wart, 1982). In contrast other authors report that porcine LAP has a K_m of 1mM, a k_{cat} of 0.92 s^{-1} and a k_{cat}/K_m of 917 $M^{-1}.s^{-1}$ for the hydrolysis of leucine *p*-nitroanilide at pH 8 (solvent not disclosed) (Van Wart and Lin, 1981). These results are difficult to compare because the experimental conditions differed; in particular the concentration of divalent cations differs, *i.e.* 1mM magnesium and 0.1mM zinc (Lin and Van Wart, 1982) and 5mM manganese and 0.1mM zinc (Van Wart and Lin, 1981) respectively. Further, the temperature and pH also differs, *i.e.* pH 9 and 23 ^{O}C (Lin and Van Wart, 1982) *vs.* pH 8 and 22 ^{O}C (Van Wart and Lin, 1981). There is also difficulty in comparing these results with those of small intestinal APN. Firstly, APN may be bound to BBMV or solubilised from the membrane by the action of proteases or detergents. Secondly, the experimental conditions may vary, *e.g.* enzyme purification, model substrate and pH. For example, BBMV-bound porcine APN has a K_m of 2.33 mM and V_{max} 9.55 μmol/min/mg (L-alanine-p-nitroalanine chloride, pH 7) (Fan et al., 2002). Whereas, triton X100 solubilised/purified APN has a K_m of 0.52 mM and a V_{max} of 59.5 μmol/min/mg (Leu-naphthylamide, pH 8) (Caporale and Troncone, 1988). These factors make it difficult to meaningfully compare the kinetics of LAP and APN.

11.1.6.1.2 C-terminal exopeptidase (carboxypeptidase)

Although the suite of pancreatic enzymes includes a number of *C*-terminal exopeptidases (carboxypeptidases), *e.g.* carboxypeptidase A (CPA) and carboxypeptidase B (CPB), they are unable to hydrolyse a number of important amino acid residues. Carboxypeptidase A and CPB hydrolyse aliphatic/aromatic and basic residues from the carboxy terminus of oligopeptides respectively (Beck, 1973). However, CPA cannot hydrolyse amino acids in the *D*-conformation, P'_1 proline, sarcosine (Stahmann et al., 1946) or charged groups (Stevens, 2006), and CPB cannot hydrolyse histidine residues (Folk and Gladner, 1958).

Further, where P'_1 proline is present P_1 amino acids are poorly hydrolysed by CPA or CPB

Two groups of BB carboxypeptidase enzymes exhibit complementary specificity to pancreatic carboxypeptidases. The first group have the capacity to hydrolyse *C*-terminal prolyl residues and the second the ability to hydrolyse acidic R-groups. Experimental evidence suggests that BB angiotensin converting enzyme (ACE, EC 3.2.1.-/3.4.15.1), and membrane carboxypeptidase P (CPP, EC 3.4.17.16) act in concert to hydrolyse *C*-terminal prolyl residues (Yoshioka et al., 1988). The second group hydrolyses acidic residues; the primary enzyme being glutamate carboxypeptidase II (GCP2, EC 3.4.17.21), hydrolyses acidic amino acids an activity distinct from pancreatic carboxypeptidases (Barinka et al., 2002). As the aforementioned small intestinal carboxypeptidases were prohibitively expensive, a yeast vacuolar enzyme was chosen to represent BB carboxypeptidases. Carboxypeptidase Y (CPY, EC 3.4.16.5) (Sigma) (Zubenko et al., 1983, Hemmings et al., 1981), purified from *Saccharomyces cerevisiae*, was chosen to represent both groups of *C*-terminal exopeptidases on the grounds of its broad specificity (IUBMB, 2013). Carboxypeptidase Y preferentially hydrolyses *C*-terminal amino acids from oligopeptides with aliphatic or hydrophobic residue in the P'_1 position, and preferably a methionine, leucine or phenylalanine in the P_1 position (The UniProt Consortium, 2014). Importantly CPY can hydrolyse proline containing peptides (Hayashi et al., 1973) and will hydrolyse aspartic acid and glycine residues, although slowly (Hayashi et al., 1973). Carboxypeptidase Y is approximately 17 % glycosylated (Zubenko et al., 1983), which may confer some protection from pancreatic proteases *in vitro*. The pH optimum of yeast CPY is approximately 7 (Bai et al., 1975), which is in keeping with

BB GC2 at pH 7 (Barinka et al., 2002, Halsted et al., 1998). Angiotensin converting enzyme has a higher pH optimum of 8.3-8.5 (Yoshioka et al., 1987).

There is no human equivalent for yeast CPY so its kinetic activity can only be compared with that of mammalian ACE and GC2, which have correlative activity profiles. Triton X100 solubilised/purified rat BB ACE has a K_m of 1.9 mM, a V_{max} of 108 μmol/min/mg, a k_{cat} of 303 s^{-1} and a k_{cat}/K_m 1.6E^5 $M^{-1}.s^{-1}$ for the hydrolysis of Benzoyl-Gly-Ala-Pro (pH 7.4) (Erickson et al., 1992). Alternately, papain solubilised/purified porcine GC2 has a K_m of 3.9E^{-3} mM and a V_{max} of 0.338 μmol/min/mg for the hydrolysis of folyl-γ-Glu-γ-[14C]Glu (pH 6.5) (Halsted et al., 1998). The K_m, k_{cat} and k_{cat}/K_m for the hydrolysis of Cbz-Gly-Phe (pH 6) by autolysed/purified carboxypeptidase Y from *Saccharomyces cerevisiae* are 1.2 mM, 2.25 s^{-1} and 1.8E^3 $M^{-1}.s^{-1}$ respectively. Hence, the activity profile of carboxypeptidase Y is not overtly different from its mammalian counterparts.

11.1.6.1.3 Endopeptidase

Neprilysin (NEP) (EC 3.4.24.11) hydrolyses the sissile bond between aliphatic (including proline) and aromatic amino acids (Hersh and Morihara, 1986). This enzyme is of interest as it's activity is distinct from the pancreatic endopeptidases (Stevens, 2006), in regards to its capacity for cleaving proline containing oligopeptides (Hersh and Morihara, 1986, Stevens, 2006). The only available source of NEP was recombinant, and thus uneconomic, hence, thermolysin (THL, EC 3.4.24.27) from *Geobacillus stearothermophylus* (Sigma) was chosen to represent endopeptidases. Thermolysin is an enzyme from the M4 peptidase family (The UniProt Consortium, 2014) that has similar specificity to NEP (bacterial source of THL not specified in this publication) (Pozsgay et al., 1986). The optimal pH for NEP is 7 (Rice et al., 2004) and the optimal pH of THL (determined by the manufacturer, Sigma Ltd)(Ooshima et al., 1985). Although the level

of glycosylation of *Geobacillus stearothermophylus* THL is not known, this enzyme is reported to be chemical and temperature stable (Vieille and Gregory Zeikus, 1996, Bruins et al., 2001). Bacterial THL binds with its substrate by hydrophobic interactions whereas NEP binds by a combination of ionic interaction, with the *C*-terminal carboxylate, and weak hydrophobic interactions (Hersh and Morihara, 1986). Both THL and NEP require a hydrophobic residue at P'_1, *i.e*. thermolysin preferentially binds to either Leu or Phe in P'_1, while NEP binds to either Phe or Tyr (Erdös and Skidgel, 1989).

There are differences in the kinetics of THL and NEP, but these indicate THL is the more active enzyme. Hence, the K_m of commercial thermolysin (Sigma) was 0.6 mM; the k_{cat} is 398.3s^{-1} and the k_{cat}/K_m is 0.678 $M^{-1}.s^{-1}$ for Z-Phe-Leu-Ala (pH 7) (Hersh and Morihara, 1986). While the K_m (0.9mM) k_{cat} (21.6s^{-1}) k_{cat} /K_m (0.0248 $M^{-1}.s^{-1}$) for solubilised and purified rat renal NEP, using the same substrate and conditions (Hersh and Morihara, 1986), indicates that the latter enzyme had a somewhat lower substrate affinity, slower throughput and lower efficiency under these experimental conditions.

11.1.6.1.4 Dipeptidase

Dipeptidase 1 (DP1, EC 3.4.13.19) is a BB exopeptidase dipeptidase and is the only known dipeptidase from the intestinal BB that is classified as such (IUBMB, 2013). Dipeptidase 1 has broad specificity, hydrolysing a wide range of dipeptides (The UniProt Consortium, 2014, Amidon and Lee, 1994, Kozak and Tate, 1982), as well as leukatriene D4 and glutathione and its conjugates (The UniProt Consortium, 2014, Kozak and Tate, 1982). No information was found regarding the glycosylation of intestinal DP1, but renal DP1 is approximately 28 % glycosylated (Hooper et al., 1990). The pH optimum of human renal DP1 (pH 7.4) (Mitsuhashi et al., 1988) is close to that of porcine intestinal BB DP1 (pH 7) (Danielsen et al., 1980b) suggesting physiological similarities. The kinetic activity of small intestinal DP1 has not been described, but those of renal DP1

have. Hence, the K_m of papain solubilised and purified rat renal DP1 is 0.8 mM and the V_{max} is 1290 μmol/min/mg when hydrolysing a *L*-Ala-Gly substrate (pH 8) (Kozak and Tate, 1982).

Recombinant human intestinal DP1 and porcine renal DP1 are prohibitively expensive making them inappropriate for incorporation at this time. Fortunately, LAP has dipeptidase activity (Kozak and Tate, 1982) and will compensate for the absence of a true dipeptidase enzyme in this array.

11.1.6.1.5 Lipolytic consortium

The number of lipolytic enzymes present in the mammalian BB is limited. Proteomic analysis of BBMV suggests that 10 % of spectral counts identified as BB hydrolases are lipolytic enzymes, and that they are limited to sphingolipid and phospholipid hydrolysing enzymes (McConnell et al., 2011). This is likely to be due to efficient lipid hydrolysis by the combined action of buccal, gastric and pancreatic lipases (Bauer et al., 2005, Bernbäck et al., 1990). As outlined previously, enzymes that fall below the threshold of 2 % dietary intake will not be included in the BB phase. The calculated intake of phospholipids is 1.3-3.3 % of ingested dietary lipid (Åkesson, 1982) this amounts to 0.44-1.12 % of daily energy intake, in the New Zealand diet, and as such did not merit inclusion. Further, as mentioned hitherto sphingolipid hydrolysing BB enzymes will not be included into the array as they do not meet the 2 % dietary threshold. Those interested in the hydrolysis of sphingolipids could add the requisite enzymes into the array as needed.

11.1.6.2 Oligosaccharidase consortium

Brush border oligosaccharidases are important enzymes for incorporation into the proxy BB *in vitro* system. This is underscored by the fact that deficiency of BB enzymes such

as SI and LPH engenders distressing, physical symptoms and a clinical syndrome that have the potential to cause nutrient insufficiency.

All four of the BB border oligosaccharidases are exoenzymes (Van Beers et al., 1995b) giving them distinct activity from the endo-enzymatic activity of pancreatic amylase (Beck, 1973, The UniProt Consortium, 2014). Most of the BB oligosaccharidases are able to catalyse the cleavage of more than one type of linkage with some having unique specificity. The BB oligosaccharidases include the α1,4 glucosidase complexes SI and MGAM, and the *β*–glucsosidase complex LPH. All three enzyme complexes have two active sites (Van Beers et al., 1995a), which is equivalent to six distinct enzymes. The fourth glucosidase, trehalase (EC 3.2.1.28), was not included in the array as its only role is in the digestion of the α-1, α-1 linkages in trehalose (Van Beers et al., 1995a). Trehalose is found in a limited range of foods, *i.e.* mushrooms, yeast and insects (Van Beers et al., 1995a), honey, lobster, prawns, mirin and sherry (Richards et al., 2002). In general 7.7-8.75 grams of trehalose is eaten per day (*i.e.* between 131-149 kj/day) based on the medium lower and upper limits of trehalose found in foods in the western diet (Abbott and Chen, 2000); *i.e.* 1.47–1.67 % of daily energy intake (University of Otago and Ministry of Health, 2011). Hence, trehalase would not warrant addition into the enzyme array. In countries where trehalose is ingested in greater amounts its addition would be warranted.

11.1.6.2.1 Sucrase–isomaltase

Sucrase-isomaltase is a highly glycosylated (~25 %) (Naim et al., 1988a) enzyme complex that is composed of two subunits each with distinctive active sites, *i.e*. sucrase (EC 3.2.1.48) and isomaltase (EC 3.2.1.10) (Semenza, 1986). The isomaltase site catalyses the hydrolysis of α-1,6-linked glucose in oligosaccharides produced from starch and glycogen, including isomaltose, isomaltulose, isomaltotriose and panose (IUBMB,

2013). Isomaltase and sucrase contribute to the hydrolysis of α-1,4-linked glucose oligomers (Gray et al., 1979) and the sucrase site also catalyses the hydrolysis of sucrose

.

Currently, the only available SI is a recombinant enzyme and is once again uneconomical for routine *in vitro* digestion. It is nevertheless important to include a sucrase degrading enzyme given that sucrose comprises 4 % of carbohydrate intake in the western diet (University of Otago and Ministry of Health, 2011). We therefore substituted invertase (EC 3.2.1.26), a β-fructofuranosidase (IUBMB, 2013) from *Saccharomyces cerevisiae* (Megazyme International), which is highly glycosylated at approximately 50 % (Neumann and Lampen, 1967) and presumably stable. The literature shows that *Saccharomyces cerevisiae* has four isoforms of β-fructofuranosidase and that all have a Km of 25.6 mM (sucrose, pH 4.5), share a pH optimum of 3.5-5 and have a temperature optimum of 60 ^{O}C (Andjelković et al., 2010). The supplier suggests the enzyme is stable from pH 3.5 to 8. The hydrolysis of sucrose by rat SI (autolysed and subsequently purified) had a K_m of 19 mM and k_{cat} of 120 s^{-1} for the hydrolysis of sucrose (pH 6.1, 37 ^{O}C).

To fully replace the actions of SI it is also necessary to include an enzyme capable of α1,6 glycosidase activity (EC 3.2.1.10) of isomaltase. Hence, a recombinant microbial oligo α1,6 glycosidase (EC 3.2.1.10) (Megazyme International) was used to replicate this activity (EC 3.2.1.10). While the enzyme is commercially available, the microbial source is commercially confidential so there are no details available regarding its kinetic activity. The supplier notes a pH optima of 4.5, but suggest oligo α1,6 glycosidase is pH stable from of 5-9.

11.1.6.2.2 Maltase – Glucoamylase

The MGAM complex consists of maltase (EC 3.2.1.20) and glucoamylase (EC 3.2.1.3). Maltase catalyses the hydrolysis of terminal, non-reducing α-1,4-linked glucose residues from glucose oligomers with release of α-D-glucose (IUBMB, 2013). The glucoamylase site catalyses the hydrolysis of terminal α-1,4-linked glucosidic bonds successively from non-reducing ends of starch side chains with release of β-D-glucose, and can release α-1,6 glucosidic bonds when the next residue is α-1,4-linked (IUBMB, 2013, Van Beers et al., 1995b). Hence, glucoamylase activity complements the α-1,6 activity of isomaltase. The only source of commercially available human MGAM is expensive recombinant enzyme and as such is not favourable. Hence, separate enzymes from microbial sources were used to replicate the actions of the maltase and glucoamylase components.

Firstly, an α-glucosidase (EC 3.2.1.20) from *Bacillus stearothermophillus* (Megazyme International) was chosen to represent maltase. It catalyses the hydrolysis of terminal α-1,4-linked D-glucose from glucose oligomers, of 2-6 residues, and will hydrolyse α-1,4-linked D-glucose in dextrins, it weakly hydrolyses the α1,β2 bonds of sucrose, but has no activity towards α-1,6-linkages (Suzuki et al., 1984). The pH optimum is 6.4 (Suzuki et al., 1984), which is consistent with the supplier's suggested pH optima of between 6 and 7 (stable pH 5-11). The K_m for maltose was 5.6 mM, while the k_{cat} and k_{cat}/K_m were 877 $^{s-1}$ and 1.57E^5 respectively (pH 6.8, 60ly w Human recombinant maltase (*N*-terminal MGAM) has a K_m of 6.17mM, a k_{cat} of 47.76 s^{-1} and a k_{cat}/K_m of 7.74E^3 $M^{-1}.s^{-1}$ for the hydrolysis of maltose (pH 7, 37^O) (Ren et al., 2011). These results indicate that although the substrate specificity is similar, but the bacterial maltase is catalytically more efficient at hydrolysing maltose than its BB counterpart. Secondly, a glucoamylase (EC 3.2.1.3) (Megazyme International), from *Hormoconis resinae* was chosen to represent glucoamylase (EC 3.2.1.3). The supplier suggests a pH optimum of 4-5 at 40 ^{O}C (stable pH 3-9). The

fungal enzyme has a Km for maltose of 14.55 mM a k_{cat} of 1.92 s^{-1} and a k_{cat}/K_m of 1.32E^2 $M^{-1}.s^{-1}$ (pH 4.3, 25 ^{O}C) (Fagerström, 1991). Human recombinant glucoamylase (*C*-terminal MGAM) has a K_m of 5.53 mM, a k_{cat} of 21.99 s^{-1} and the k_{cat}/K_m is 3.98E^3 $M^{-1}.s^{-1}$ for the hydrolysis of maltose at pH 7 and 37^{O}C (Ren et al., 2011). In this instance human glucoamylase is catalytically more efficient at hydrolysing maltose than the glucoamylase yeast equivalent.

11.1.6.2.3 Lactase - phlorizin hydrolase

Lactase-phlorizin hydrolase is a *β*-glycosidase complex consisting of two sites. The lactase (EC 3.2.1.108) site hydrolyses lactose and cellobiose/triose; has slow action towards cellulose; and displays some activity towards aryl *β*-glycosides (Semenza, 1986). The phlorizin site (EC 3.2.1.62), a glycosylceramidase, hydrolyses aryl *β*-glycosides, glyceroceramides (Semenza, 1986), phorizin and lactase (IUBMB, 2013). The kinetic activity of triton X100 solubilised and purified rat LPH is K_m 16 mM, k_{cat} 47 S^{-1}, k_{cat}/K_m 2.9E^3 $M^{-1}.s^{-1}$ (lactose, pH 6 and 37 ^{O}C) .

There appears to be no substitute for phlorizin hydrolase and the human recombinant LPH is not an economic alternative. Hence, only the *β*-glucosidase activity of lactase will be replicated *in vitro* by enzymes from other sources. A recombinant *β*-glucosidase (EC 3.2.1.21) from *Agrobacterium sp.* (Megazyme) was chosen. It hydrolyses terminal non-reducing *β*-D-glucosyl residues with release of β-D-glucose (IUBMB, 2013). The pH optimum of this enzyme is 6.5–7 (stable from pH 5-9). As the species of *Agrobacterium* is not indicated the activity given below is that provided by the supplier (175 U/mg protein; *p*-nitrophenyl *β*-D-glucopyranoside, pH 6.5, 40^{o}C); the kinetic activity being unknown.

11.2 Experimental methodology

11.2.1 Chemicals

Where possible the chemicals purchased were of premium or analytical grade. Glucose standard (G6918), L-Proline (P5607), 4-Nitroaniline (N2128), Mannitol (M4125), L-*N*-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (S8511), L-leucine-*p*-nitroanilide (L9125), Isomaltotriose (I0381), Soluble starch (S9765), Maltose (potato) (M-5885), phosphoramidon (R7385) and β-Lactose (L3750) were from Sigma – Aldrich. Sucrose (530) was from Ajax chemicals, Bz-Tyr- *p*-nitroanilide (L-1155.0001) from Bachem, and GOPOD (K-GLUC) from Megazyme International.

11.2.2 Enzymes

Carboxypeptidase Y (*Sacchromyces cerevisiae*) (C3888), Leucine aminopeptidase (porcine kidney) (L6007), and Thermolysin *(Geobacillus stearothermophylus)* (T7902) were purchased from Sigma–Aldrich. Oligo-1,6-glucosidase (recombinant microbial) (E-OAGUM), and α-glucosidase (*Bacillus stearothermophilus*) (E-TSAG) Glucoamylase P (E-GAMP), Invertase (*Sacchromyces cerevisiae)* (E-INVPD2) and β-glucosidase (*Agrobacterium sp.*) (E-BGOSAG) were purchased from Megazyme International.

11.2.3 Method

The kinetic activity of the enzymes were determined individually, in triplicate, at pH 6 (oligosachharidases) or 8 (oligopeptidases). Nine substrates were chosen to individually characterise the activity of these enzymes. Concomitantly the hydrolysis of these substrates was determined with the enzymes in admixture. Note: although we endeavoured to use substrates that reflect the unique specificity of the component enzymes there is common substrate specificity, as mentioned hitherto, which was unavoidable. The pH at which individual and admixed enzymes were assayed was chosen

to be in the operational pH range of component enzymes (Figure 41). The proportions of enzymes were in keeping with the proportions used in the *in vitro* experiments, but modified to ensure that the enzyme activity was within the straight part of the standard curve.

11.2.4 Conversion method

The enzyme assays were optimised and then the information garnered was used to choose the enzyme proportions that were going to be used in an i*n vitro* system. The method used was based on the enzymes with lowest and highest proportion of spectral counts within each consortia. As mentioned in section 1.2.4 spectral counts are not a true reflection of enzyme *in vivo* as larger proteins (more peptide fragments) elicit more spectra and some peptide fragments are more readily identified. However, it provided the best estimate currently available.

The proportion of enzymes within each consortia were calculated separately. A conversion factor was calculated for each enzyme, *e.g.* Oligopeptidase consortia: LAP (highest) divided by THL (lowest) = 1346/379 = 3.55. A separate conversion factor was then established for all of the other enzymes as a proportion compared to THL, *e.g.* CPY= 523/379 = 1.38. New concentrations were then established by multiplying by the conversion factor required by the enzyme with lowest enzyme proportions *e.g.* LAP 3.55*0.033 (THL).

Table 52. Proportions and concentrations of oligopeptidases to be added to the brush border phase

Group	Enzyme	Proxy	Spectral count	Concentration of enzyme in the characterisation assay (µg/µl)	Final concentration of enzyme in characterisation assay (µg/µl)	Conversion factor	Initial concentration of enzyme (µg/µl)	Concentration of enzyme in *in vitro* system (µg/µl)
oligopeptidase	APN	LAP	1346	0.0125	0.00625	3.5514512	0.1172	0.0586
oligopeptidase	NEP	THL	379	0.033	0.0165	1	0.0330	0.0165
oligopeptidase	ACE + GC2	CPY	523	0.25	0.125	1.379947	0.0455	0.02275

Table 53. Proportions and concentrations of oligosaccharidases to be added to the brush border phase

Group	Enzyme	Proxy	spectra	Concentration of enzyme added to characterisation assay (µg/µl)	Final concentration of enzyme in characterisation assay (µg/µl)	Conversion factor	Calculated: Initial concentration of enzyme for *in vitro* (µg/µl)	Final concentration of enzyme in *in vitro* system (µg/µl)
oligosaccharidase	isomalt	oligo 1,6	699	0.0002	0.0001	2.45263	0.002	0.001
oligosaccharidase	sucrase	invert	699	0.00025	0.000125	2.45263	0.002	0.001
oligosaccharidase	maltase	α-gluc	610	0.0005	0.00025	2.14035	0.0017	0.00085
oligosaccharidase	lactase	β-gluc	285	0.0008	0.0004	1	0.0008	0.0004
oligosaccharidase	gluco	amylo	610	0.005	0.0025	2.14035	0.0017	0.00085

11.2.5 Characterisation assays

Enzymes were assayed alone and in admixture.

11.2.6 Analysis

The kinetic activity of LAP, CPY, THL, α-glucosidase, amyloglucosidase, invertase, oligo-1, 6-glucosidase and β-glucosidase were estimated using non-linear fit and Origin 8.5 software.

11.3 Results

There was no time to finish the last few enzyme assays for this chapter. Time constraints were put on the assaying of enzymes, as all enzymes needed to be used within 4 hours. Where enzymes were assayed alone and in admixture the results are given.

11.3.1 Optimisation assays

11.3.1.1 β-glucosidase

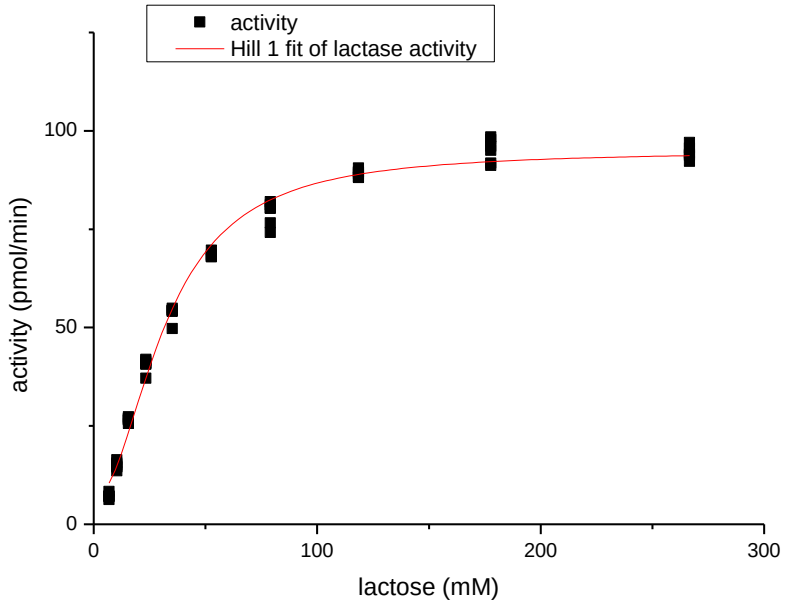

Figure 35. Hill 1 fit of β-glucosidase activity

- 18.1.13 Fresh: K_m 33 mM (s.e. 2.56) V_{max} 103.42 pmol/min (s.e. 4.18) r^2 0.97
- 13.9.14 Fresh: K_m 30 mM (s.e. 1.79) V_{max} 134 pmol/min (s.e. 2.39) R^2 0.98
- 13.10.14 Fresh: K_m 32 mM (s.e. 0.9) V_{max} 88.45 pmol/min (s.e. 1.3) R^2 0.99:
-

Table 54.Hill 1 fit of βglucosidase activity (associated with figure 35)

Model	Hill1		
Equation	y = START + (END - START) * x^n / (k^n + x^n)		
Reduced Chi-Sqr	9.00542		
Adj. R-Square	0.99114		
		Value	Standard Error
activity	START	6.49636	1.04717
activity	END	94.95287	0.76854
activity	k	32.05868	0.90247
activity	n	2	0

11.3.1.2 Oligo 1,6 glucosidase

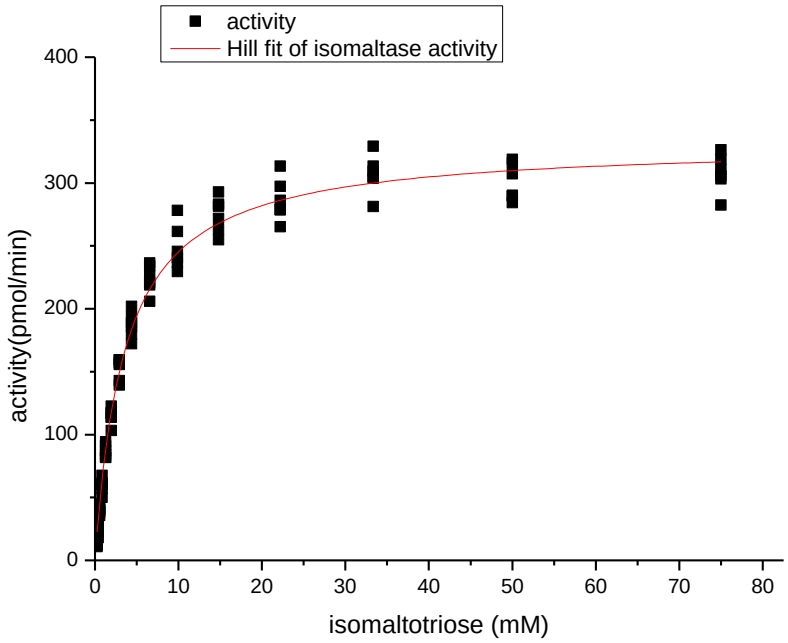

Figure 36.Hill 1 fit of oligo 1,6 glucosidase activity

- 13.9.14: K_m 1.28 mM (s.e. 0.062) and V_{max} 247 pmol/min (s.e. 3.58) R^2 0.98
- 13.10.14: K_m 1.76 mM (s.e. 0.0.063) and V_{max} 332 pmol/min (s.e. 3.15) R^2 0.99.

Table 55.Hill 1 fit of oligo 1,6 glucosidase activity (associated with figure 36)

Model	Hill		
Equation	y=Vmax*x^n/(k^n+x^n)		
Reduced Chi-Sqr	151.83631		
Adj. R-Square	0.98716		
		Value	Standard Error
activity	Vmax	331.6752	3.15125
activity	k	1.7628	0.06306
activity	n	1	0

11.3.1.3 Amyloglucosidase

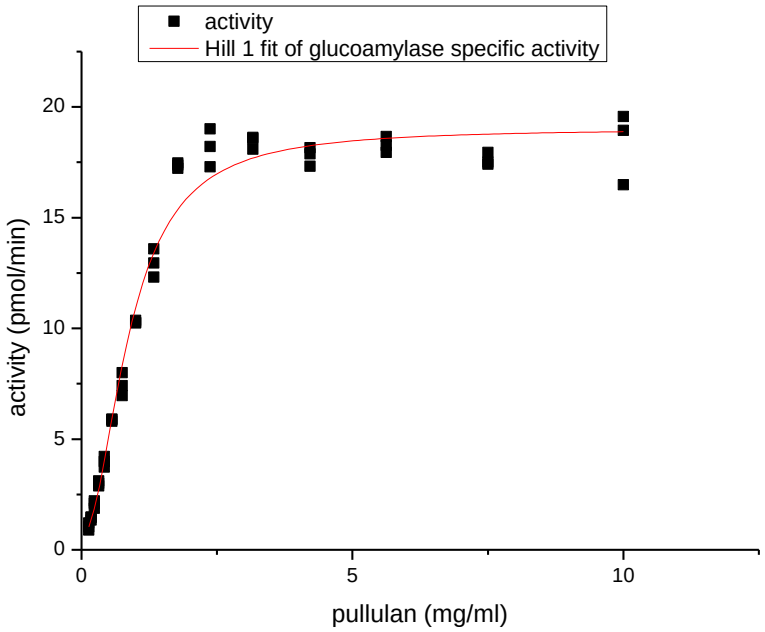

Figure 37.Hill 1 fit of glucoamylase specific activity

- 13.10.14 Fresh: K_m 0.88mM (s.e. 0.04) V_{max} 18.39 pmol/min (s.e0.41) ^{r2} 0.98.

Table 56.Hill 1 fit of glucoamylase specific activity (associated with figure 37)

Model	Hill1		
Equation	y = START + (END - START) * x^n / (k^n + x^n)		
Reduced Chi-Sqr	0.86421		
Adj. R-Square	0.9825		
		Value	Standard Error
activity	START	0.63006	0.32005
activity	END	19.02467	0.25439
activity	k	0.88402	0.0397
activity	n	2	0

11.3.1.4 Invertase

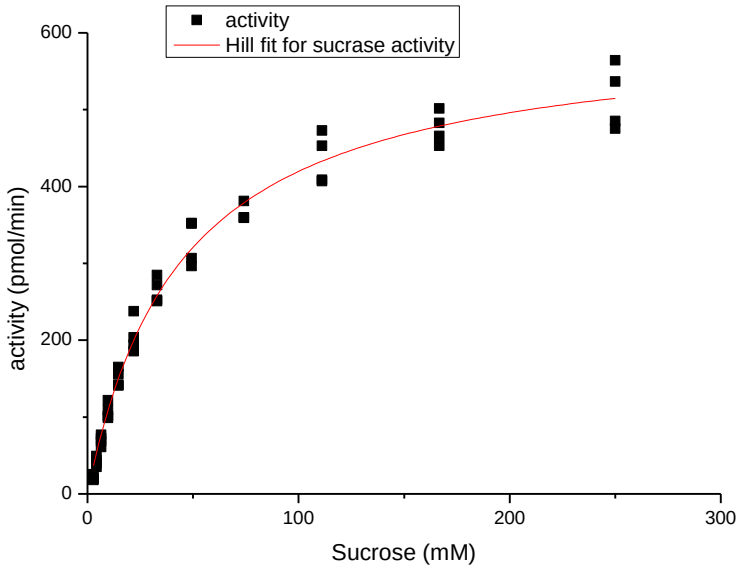

Figure 38. Hill fit of invertase activity

- 13.9.14 Fresh: K_m 42 mM (s.e. 2.96) V_{max} 581 pmol/min (s.e. 14.6) r^2 0.97.
- 13.10.14 Fresh: K_m 45 mM (s.e. 2.52) V_{max} 606 pmol/min (s.e 12.51) ^{r2} 0.99.

Table 57. Hill fit of invertase activity (associated with figure 38)

Model	Hill		
Equation	y=Vmax*x^n/(k^n+x^n)		
Reduced Chi-Sqr	423.34623		
Adj. R-Square	0.98552		
		Value	Standard Error
activity	Vmax	606.4202	12.51373
activity	k	44.50444	2.51964
activity	n	1	0

11.3.1.5 1,4 glucosidase

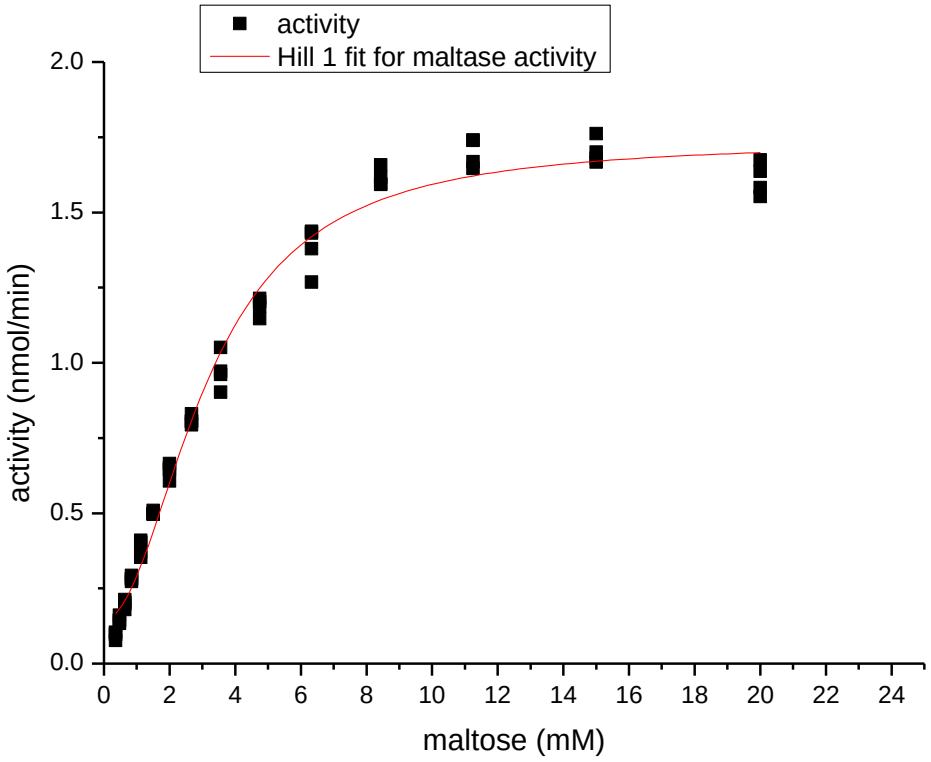

Figure 39. Hill 1 fit for 1,4 glucosidase activity

- 13.10.14 Fresh: K_m 3.16 mM (s.e. 0.21) V_{max} 1.59 nmol/min (s.e. 0.02) r^2 0.99. (10 min)

Table 58. Hill 1 fit for 1,4 glucosidase activity (associated with figure 39)

Model	Hill1		
Equation	y = START + (END - START) * x^n / (k^n + x^n)		
Reduced Chi-Sqr	0.00431		
Adj. R-Square	0.98783		
		Value	Standard Error
activity	START	0.14663	0.01717
activity	END	1.73766	0.02014
activity	k	3.16275	0.10459
activity	n	2	0

11.3.1.6 Leucine aminopeptidase

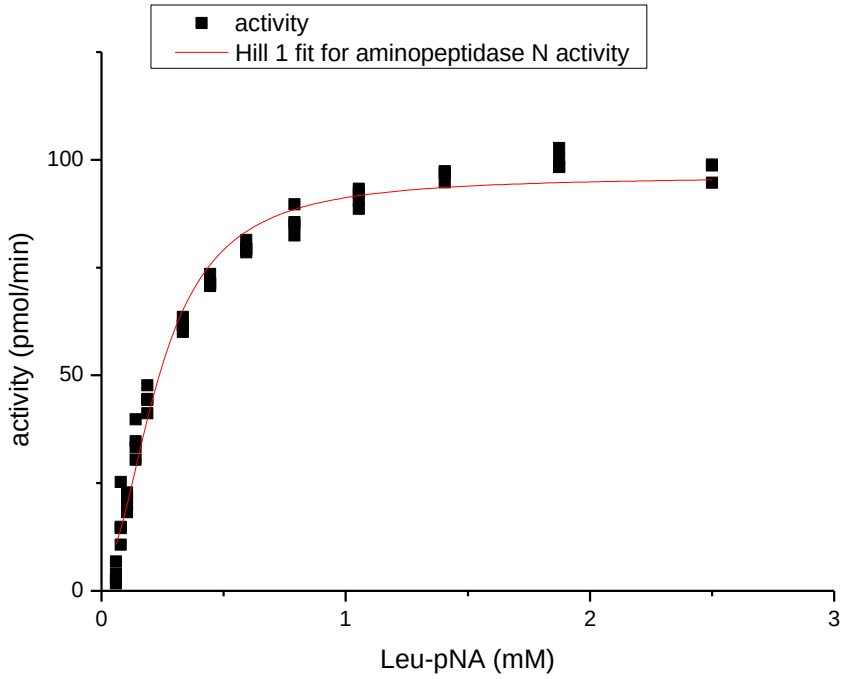

Figure 40. Hill 1 fit of leucine aminopeptidase activity

- 20. 10.14: K_m 0.24 mM (s.e. 0.013) and V_{max} 90.65 µmol/min (s.e. 2.40) R^2 0.98 (30 minutes)

Table 59.Hill 1 fit of leucine aminopeptidase activity (associated with figure 40)

Model	Hill1		
Equation	y = START + (END - START) * x^n / (k^n + x^n)		
Reduced Chi-Sqr	22.80228		
Adj. R-Square	0.9791		
		Value	Standard Error
activity	START	5.52091	2.037
activity	END	96.16654	1.27229
activity	k	0.24027	0.01286
activity	n	2	0

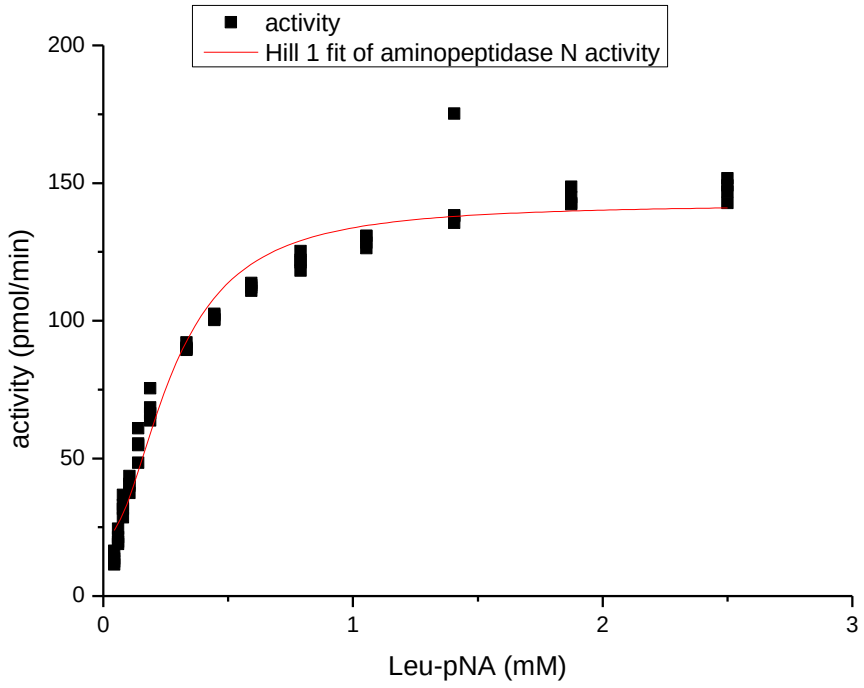

Figure 41. Hill 1 fit of leucine aminopeptidase activity (1 hr)

Table 60. Hill 1 fit of leucine aminopeptidase activity (1 hr)

Model	Hill1		
Equation	y = START + (END - START) * x^n / (k^n + x^n)		
Reduced Chi-Sqr	79.15059		
Adj. R-Square	0.96491		
		Value	Standard Error
activity	START	20.82933	2.78341
activity	END	142.4621	2.35818
activity	k	0.27794	0.01815
activity	n	2	0

11.3.1.7 Thermolysin

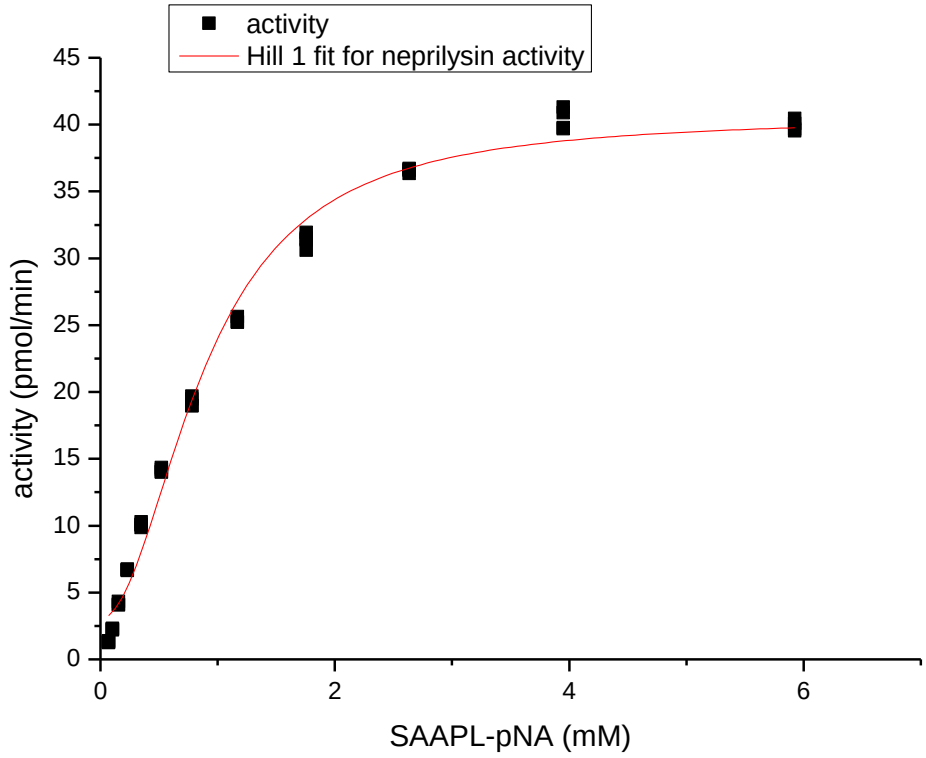

Figure 42. Hill 1 fit for thermolysin

Table 61.Hill 1 fit for thermolysin

Model	Hill1		
Equation	y = START + (END - START) * x^n / (k^n + x^n)		
Reduced Chi-Sqr	1.97874		
Adj. R-Square	0.99057		
		Value	Standard Error
activity	START	3.05747	0.36913
activity	END	40.59161	0.48014
activity	k	0.88973	0.02948
activity	n	2	0

- 13.9.14: K_m 2.38 mM (s.e. 0.101) and V_{max} 72 µmol/min (s.e. 1.09) R^2 0.99 (1hr)
- 14.10.14: K_m 0.89 mM (s.e. 0.029) and V_{max} 37.53 pmol/min (s.e. 0.606) R^2 0.99 (6hr)

11.3.2 Enzymes measured alone and in admixture

11.3.2.1 Oligo 1,6 glucosidase

The non-linear curves fitted to the activities of O16G, assayed alone or in admixture, are depicted in figure 42. The rates of the reactions are similar at low enzyme concentrations, but activity diverges above 1mM. Following this activity diverges with the activities of O16G in admixture hydrolysing isomaltotriose at a faster rate than O16G alone.

An unpaired t-test was conducted to test the differences in the kinetic activity of O16G measured alone or in admixture. There was not a significant difference in the calculated K_m values for O16G (d.f. 1,76, t=0.636 p=0.5497), table 62.

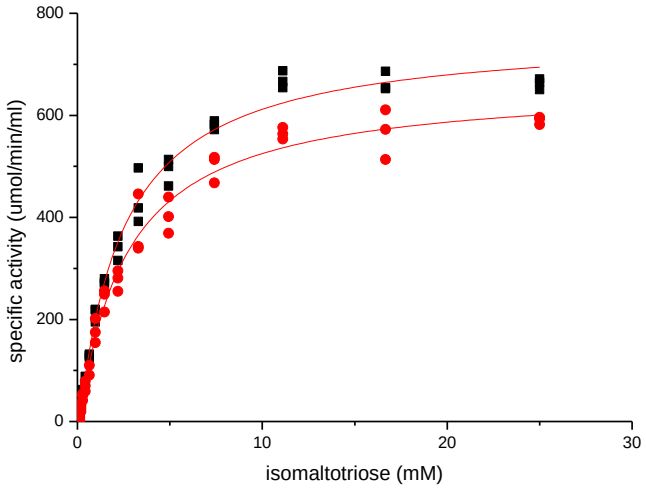

Figure 43. Comparison of the hydrolytic activity of oligo 1,6 glucosidase alone (red) and in admixture (black). The red lines fitted to the data indicate the non-linear curve fit.

However, there was a significant difference in the values calculated for V_{max} (d.f. 1,76, t=4.1690 p=0.0001). The activity of O16G being 1.15 fold greater when the enzyme was in admixture.

Table 62. Comparison of the hydrolytic activity of Oligo 1, 6, glucosidase measured alone and in admixture with other enzymes

Assay	Enzyme	Substrate	K_m (mM)	V_{max} (µmol/min/ml)	Model	R^2 value
Alone	oligo	isomaltotriose	2.51±0.24	691±18.70[a]	Hill 1	0.983
Admixture	oligo	isomaltotriose	2.34±1.69	795±16.48[b]	Hill 1	0.990

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of Vmax used the Hill 1 equation:

y = START + (END - START) * x^n / (k^n + x^n); information following the K_m and V_{max} values represents the standard error; *10mM phosphate buffer (with added cations), pH 6.* K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by Unpaired T-test (α=0.05).

11.3.2.2 Invertase

The non-linear curves fitted to the activities of invertase, assayed alone or in admixture, are depicted in figure 43. Again the activity invertase was augmented when the enzyme was in admixture.

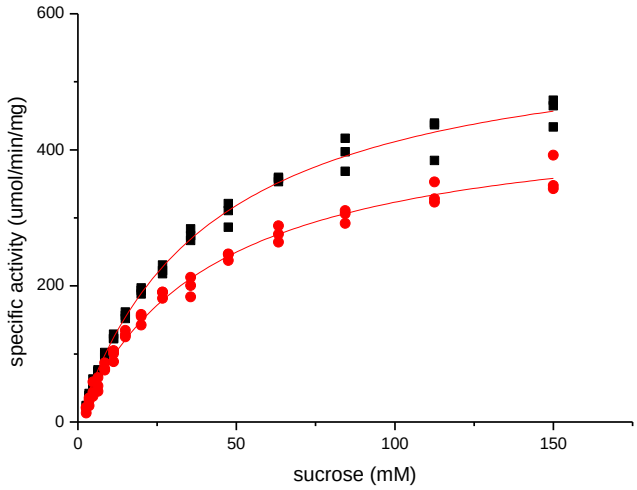

Figure 44. Comparison of the sucrose hydrolysis activity of invertase measured alone (red) and in admixture (black). The red lines fitted to the data indicate the non-linear curve fit

An unpaired t-test was conducted to test the differences in the kinetic activity of invertase measured alone or in admixture. There was no significant difference in the calculated K_m values for invertase (d.f. 1,76, t0.0463 p=0.9632), table 63. However, there was a significant difference in the values calculated for V_{max} (d.f. 1,76, t=12.8998 p=0.0001), the hydrolytic activity of invertase being 1.28 fold greater when the enzyme was in admixture.

Table 63. Comparison of the hydrolytic activity of invertase measured alone and in admixture with other enzymes

Assay	Enzyme	Substrate	K_m (mM)	V_{max} (µmol/min/mg)	Model	R^2 value
Alone	invertase	sucrose	42±2.08	458±9.66[a]	Hill 1	0.990
Admixture	invertase	sucrose	42±1.73	584±10.22[b]	Hill 1	0.993

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of Vmax used the Hill 1 equation:

y = START + (END - START) * x^n / (k^n + x^n); information following the K_m and V_{max} values represents the standard error*; 10mM phosphate buffer (with added cations), pH 6.* K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by Unpaired T-test (α=0.05).

11.3.2.3 β-glucosidase

The hydrolysis of lactose by *β*-glucosidase alone or in admixture with other enzymes are represented in figure 44. Again the activity of *β*-glucosidase is augmented in admixture, but the same maximal activity is reached.

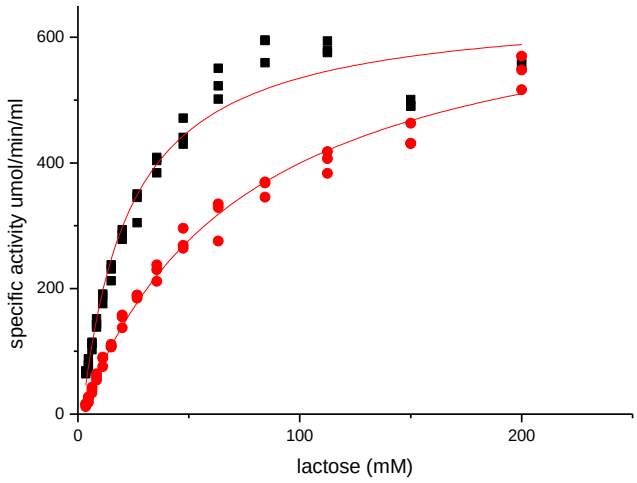

Figure 45. Comparison of the hydrolytic activity of β-glucosidase measured alone and in admixture with other enzymes

An unpaired t-test was conducted to test the differences in the kinetic activity of lactase measured alone or in admixture. There was a significant difference in the calculated K_m values for *β*-glucosidase (d.f. 1,82, t=6.9728 p<0.0001), table 64. However, there was no significant difference in the values calculated for V_{max} (d.f. 1,82, t=0.0107 p=0.9915).

Table 64. Comparison of the hydrolytic activity of β-glucosidase measured alone and in admixture with other enzymes

Assay	Enzyme	Substrate	K_m (mM)	V_{max} (µmol/min/mg)	Model	R^2 value
Alone	*β-glucosidase*	lactose	73±7.08	716±26.47	Hill 1	0.987
Admixture	*β-glucosidase*	lactose	20±2.92	716±33.11		0.965

Values obtained using the non-linear curve fit of Origin software: The model used for the estimation of Vmax used the Hill 1 equation:

y = START + (END - START) * x^n / (k^n + x^n); information following the K_m and V_{max} values represents the standard error;, K_m or V_{max} values that have different superscripted letters are significantly different from each other, as determined by Unpaired T-test (α=0.05)

12 Appendix 3.

The experimental work outlined in appendix 3 is the LC-MS data for the digestion of polyphenolics using proxy brush border enzymes (commercial enzyme extracts) using the rationale outlined in appendix 2.

12.1 Digestion of onion flesh polyphenolic extract by BBMV and PBB

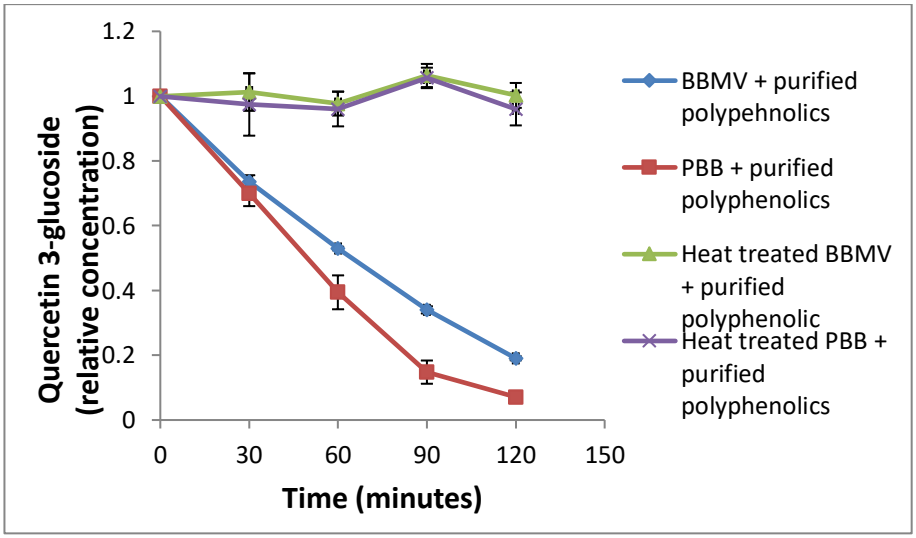

Figure 46. The relative concentration of quercetin 3-O-glucoside during a 2 hour *in vitro* digestion of purified polyphenolic compounds, with active BBMV or PBB enzyme or their equivalent heat treated controls

- These results confirm that there was a mistake made during first experiment.
- This was the positive control
- The pH change at 60 minutes does not significantly affect the digestion of plant polyphenolic compounds

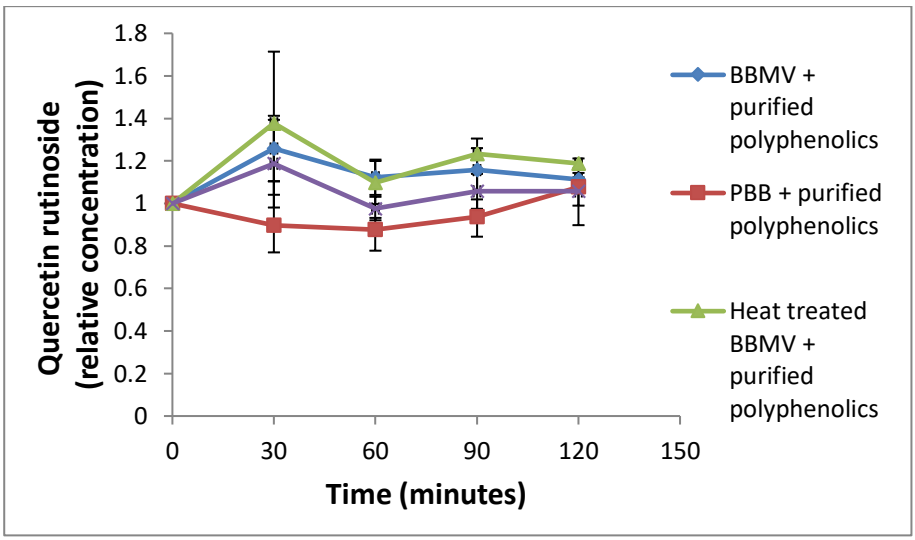

Figure 47. The relative concentration of quercetin 3-O-rutinoside during a 2 hour *in vitro* digestion of purified polyphenolic compounds, with active BBMV or PBB enzyme or their equivalent heat treated controls

- This is the negative control
- This is consistent with previous results, *i.e.* there appears to be no hydrolysis

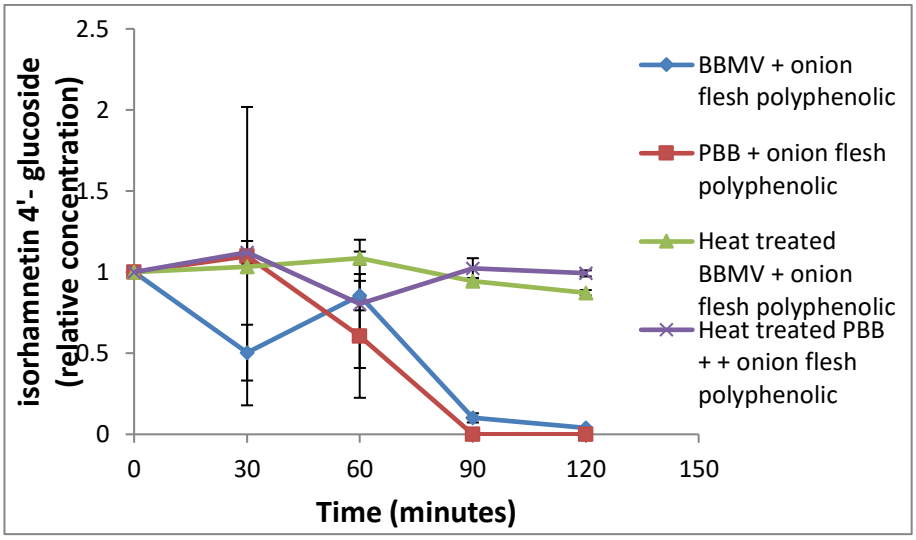

Figure 48. The relative concentration of isorhamnetin-4'-glucoside during a 2 hour *in vitro* digestion of onion flesh polyphenolic compounds, with active BBMV or PBB enzyme or their equivalent heat treated controls

- The digestion of isorhamnetin-4'-glucoside follows a similar pattern to q-3-glucoside, *i.e.* there is no pH effect
-

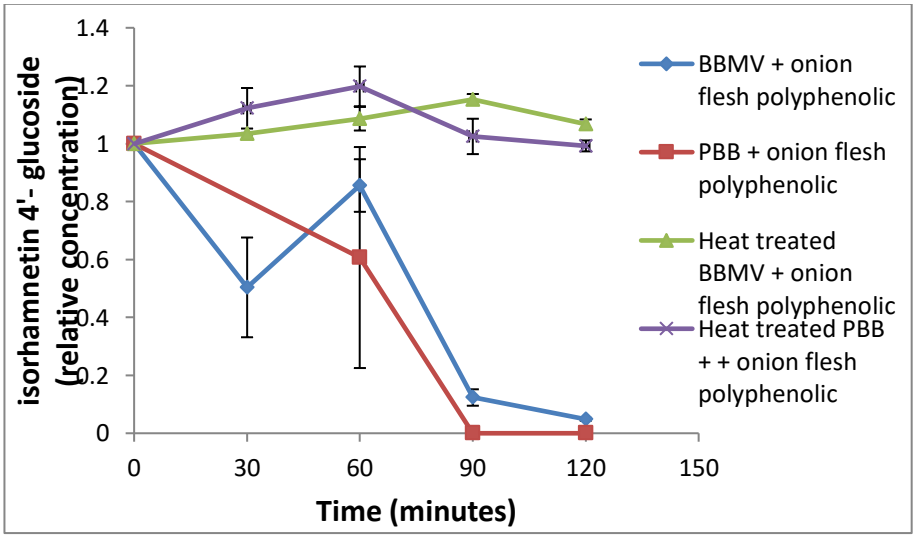

Figure 49. Repeat plotting of figure 48, with the erroneous samples removed

- Still not great... not sure why everything was so variable. Unless storage caused changes. Samples were stored in a 96 well plate and samples were aliquotted into LC-MS vials as required

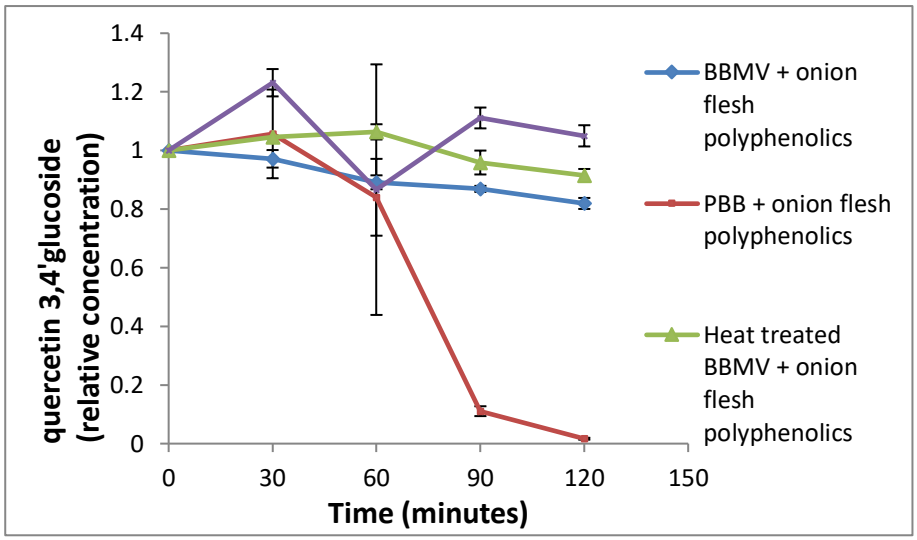

Figure 50. The relative concentration of quercetin-3,4'-glucoside during a 2 hour *in vitro* digestion of onion flesh polyphenolic compounds, with active BBMV or PBB enzyme or their equivalent heat treated controls

- The large error bars on the heat treated PBB samples at 90 minutes may be as a result of human error as the error is maintained in the other samples.

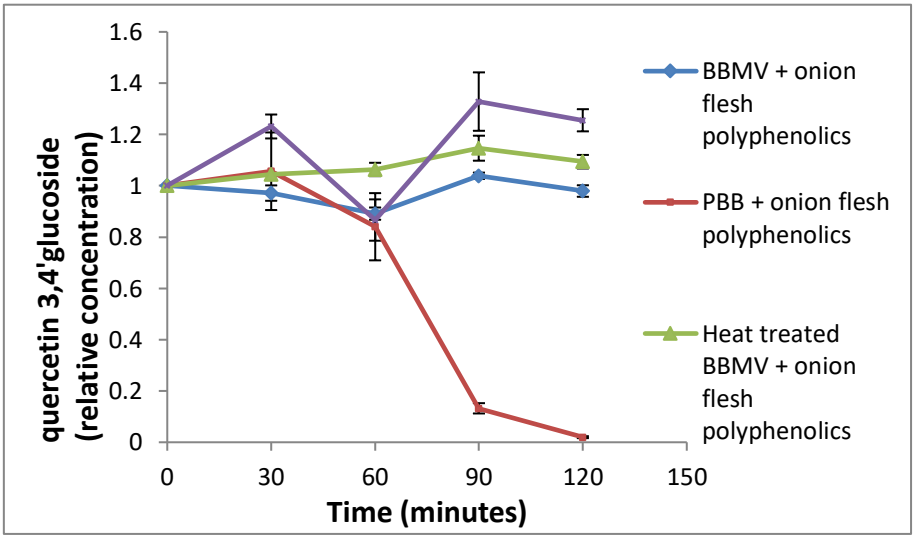

Figure 51. Repeat plotting of figure 50, with the erroneous sample removed

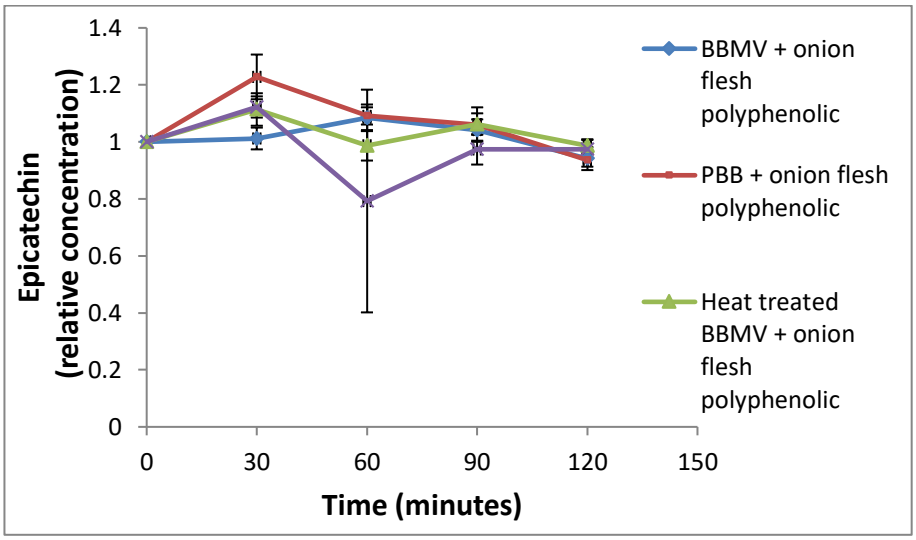

Figure 52. The relative concentration of epicatechin (reference compound) during a 2 hour *in vitro* digestion of onion flesh polyphenolic compounds with active BBMV or PBB enzyme or their equivalent heat treated controls

- This is the reference compound.
- 100 µl of 5 µg/ml epicatechin (in methanol) was added at the end of the experiment final concentration 2.5 µg/ml (Same goes for figure 53)
-

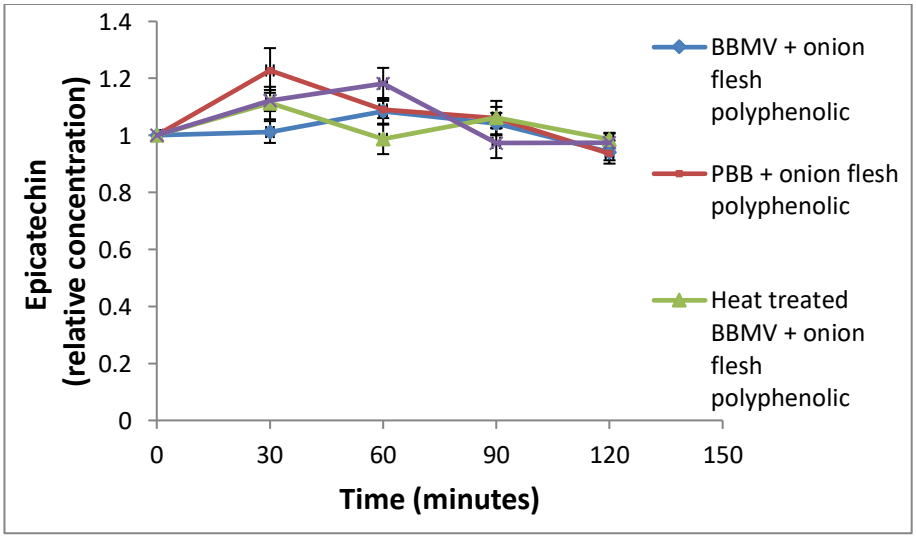

Figure 53. Repeat plotting of figure 52, with the erroneous sample removed

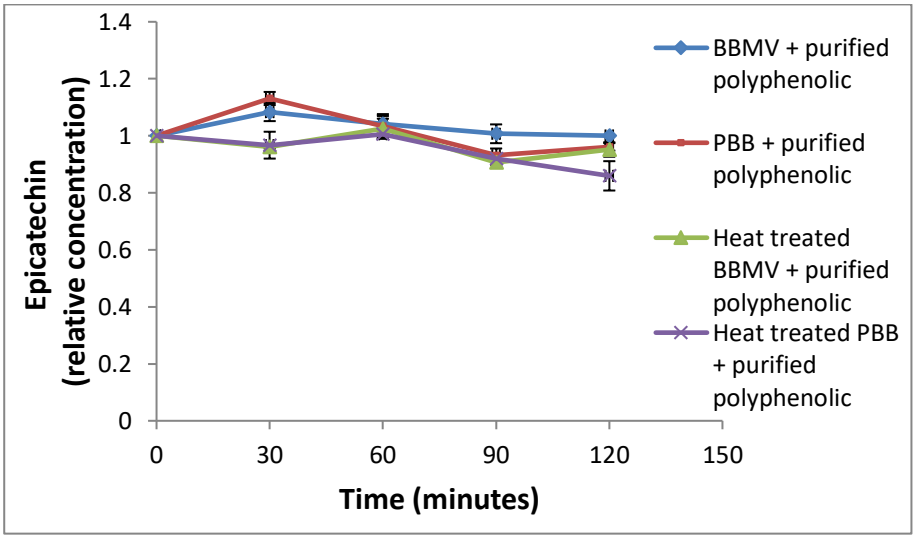

Figure 54. The relative concentration of epicatechin (reference compound) during a 2 hour *in vitro* digestion of purified polyphenolics, with active BBMV or PBB enzyme or their equivalent heat treated controls

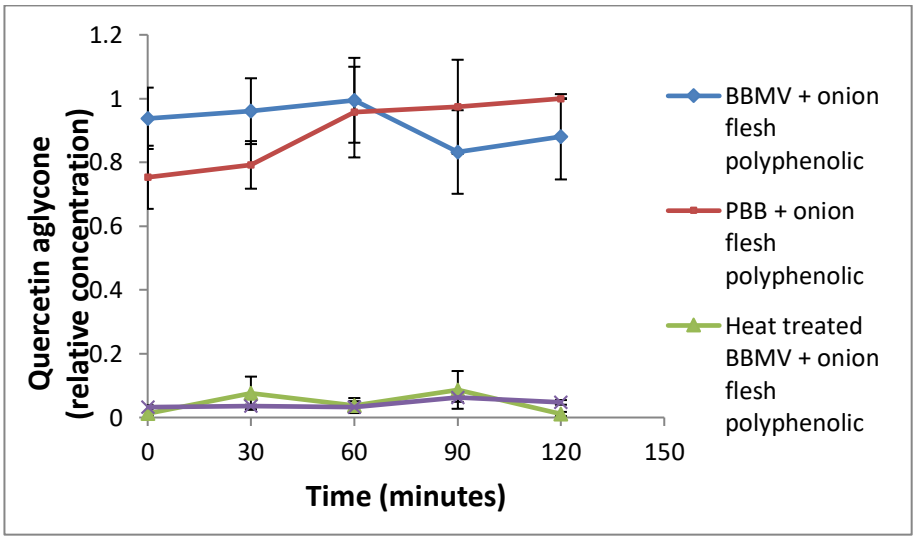

Figure 55. The relative concentration of quercetin aglycone during a 2 hour *in vitro* digestion of onion flesh polyphenolic compounds, with active BBMV or PBB enzyme or their equivalent heat treated controls

- I think that the β-glucosidases of the BBMV and PBB enzymes work very quickly, i.e. I am not quick enough to get a real time zero.
- Also there may be some degree of transglycosylation or the aglycone gets further broken down so as not to be identified as aglycone, *i.e.* by molecular weight and retention time.
- Thought: since there is more quercetin substrates to hydrolyse there may be more aglycone than the LC-MS may be able to detect. Tony said concentrations of 2-20 µg/ml are appropriate. If most of the quercetin glycosides are hydrolysed the concentration likely be in excess of 10 µg/ml.

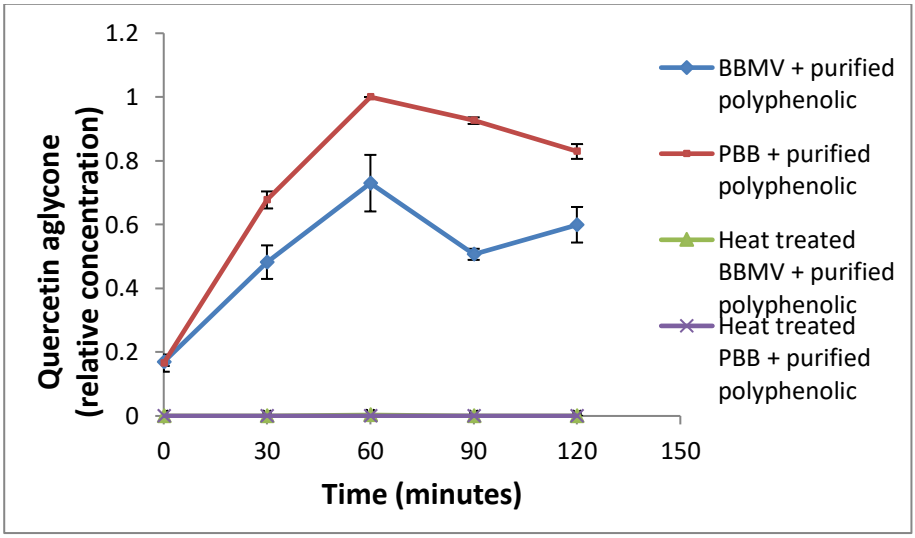

Figure 56. The relative concentration of quercetin aglycone during a 2 hour *in vitro* digestion of purified polyphenolics, with active BBMV or PBB enzyme or their equivalent heat treated controls

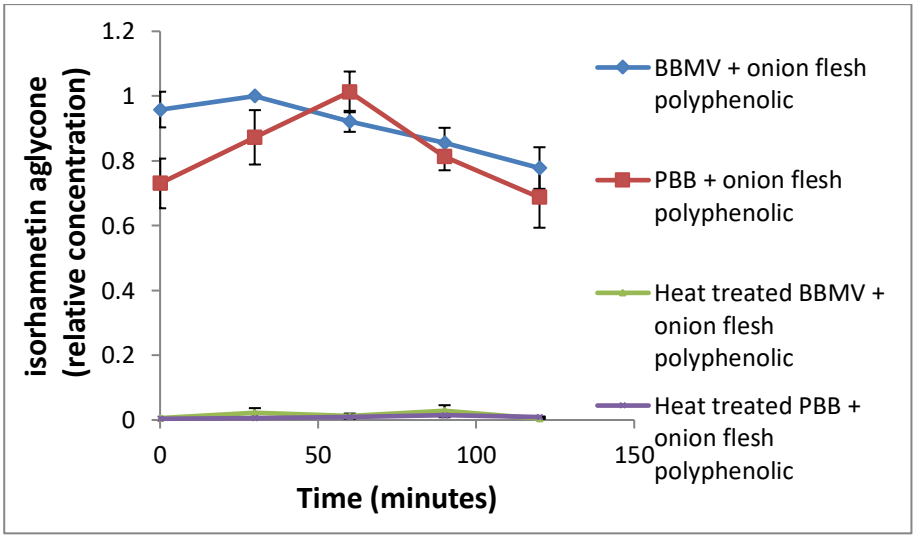

Figure 57. The relative concentration of isorhamnetin aglycone during a 2 hour *in vitro* digestion of purified polyphenolics, with active BBMV or PBB enzyme or their equivalent heat treated controls

12.2 The affect of biliopancreatic secretions on the digestion of purified polyphenolic compounds

- This work was undertaken in November and analysed in early January

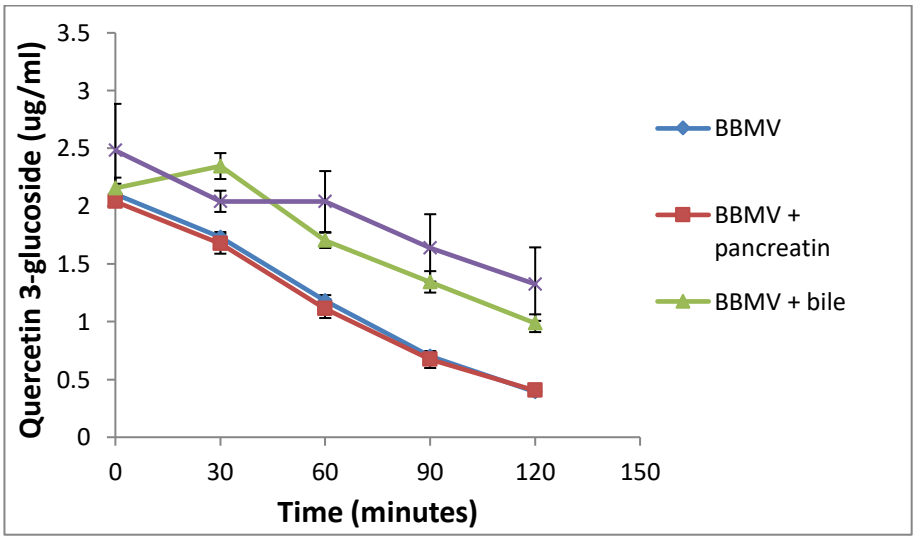

Figure 58. The concentration of quercetin 3-O-glucoside during a 2 hour *in vitro* digestion with active BBMV enzyme alone or with bile and/or pancreatin

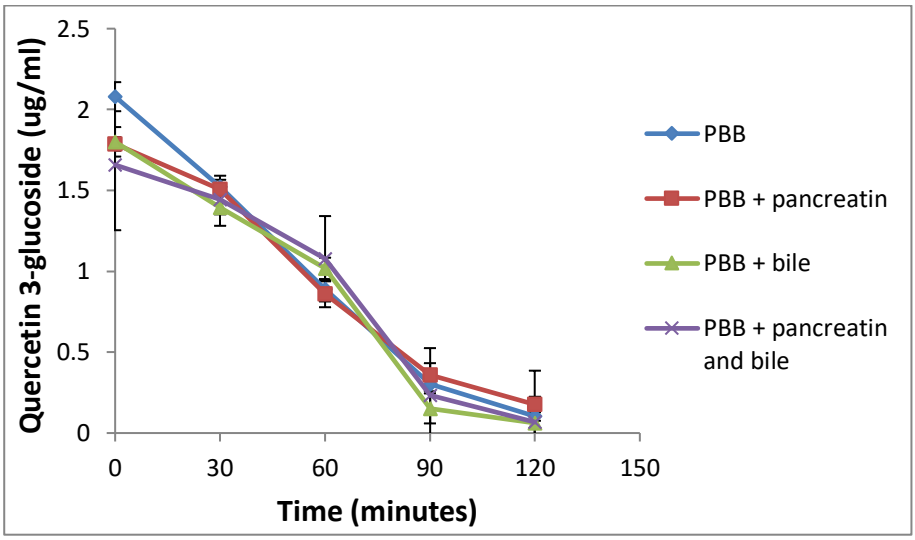

Figure 59. The concentration of quercetin 3-O-glucoside during a 2 hour *in vitro* digestion with active PBB enzyme alone or with bile and/or pancreatin

- This in vitro digestion was undertaken at pH 6 for the first hour and then pH 8 for the last hour. The PBB slope is quite different to that of the first run, *i.e.* there is activity at pH 6
- I am wondering whether I made a mistake, e.g. accidentally using heat treated enzyme instead of active enzyme in the first trial
- I will know when the next repeat comes this week – this repeat confirmed that the first set of data was erroneous.

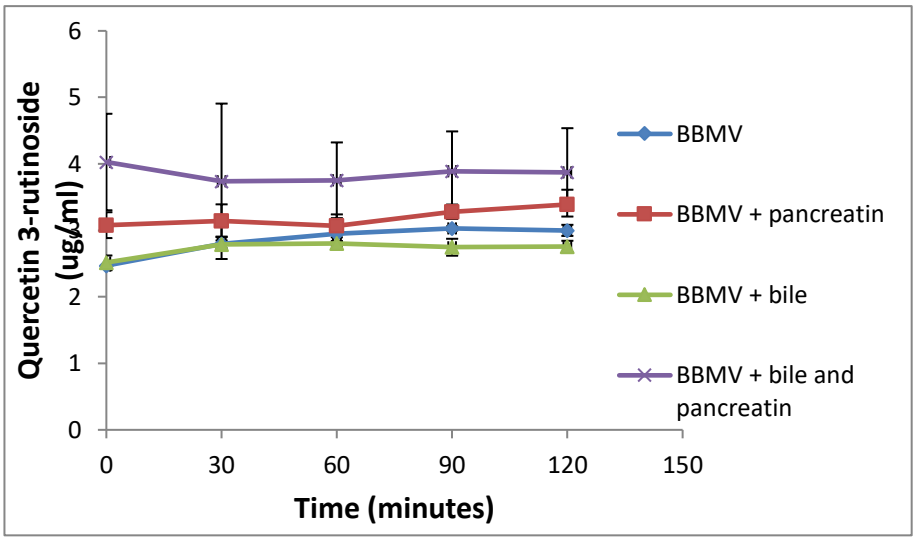

Figure 60. The concentration of quercetin 3-O-rutinoside during a 2 hour *in vitro* digestion with active BBMV enzyme alone or with bile and/or pancreatin

- Negative control

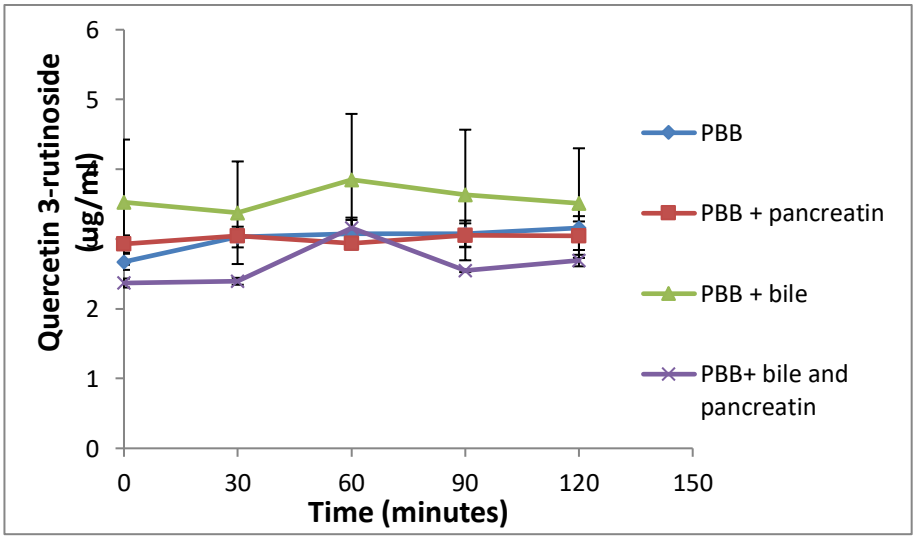

Figure 61. The concentration of quercetin 3-O-rutinoside during a 2 hour *in vitro* digestion with active PBB enzyme alone or with bile and/or pancreati

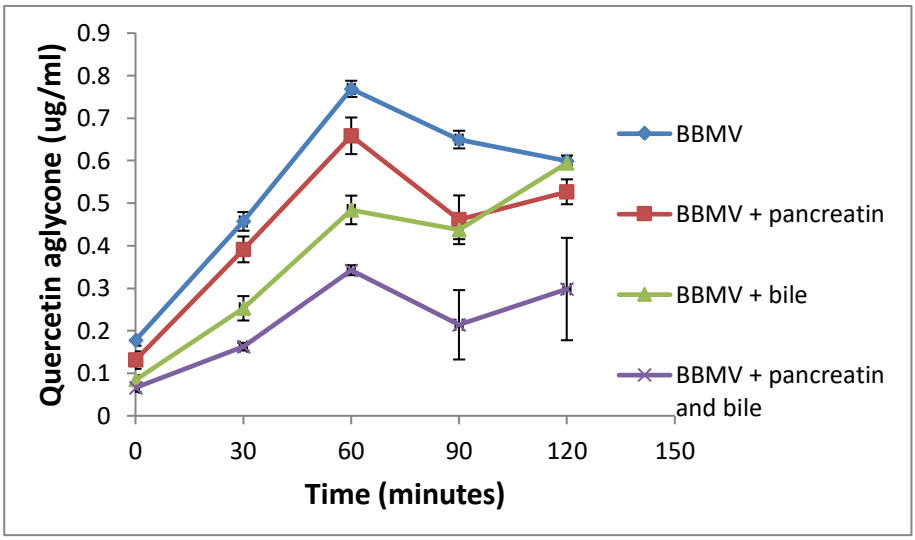

Figure 62. The concentration of quercetin aglycone during a 2 hour *in vitro* digestion with active BBMV enzyme alone or with bile and/or pancreatin

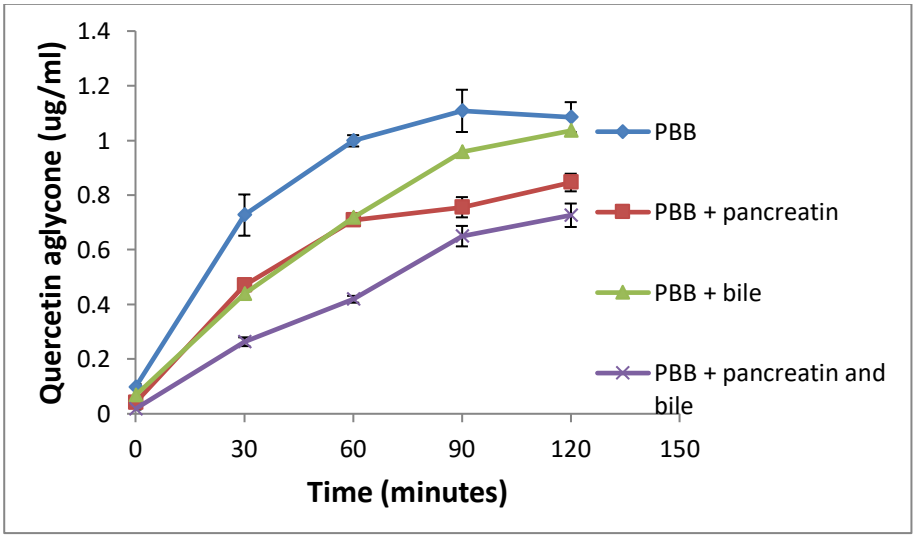

Figure 63. The concentration of quercetin aglycone during a 2 hour *in vitro* digestion with active PBB enzyme alone or with bile and/or pancreatin

- I am pleased with these results as neither bile nor pancreatin alter the degradative profiles of β-glucosidase in the PBB admixture.

13 Appendix 4.

13.1 Pilot studies

Animal data

The physical data for four sets of rats are detailed in table 65. Analysis of variance was used to assess whether group had an effect on mucosal weight (adjusted for the co-variant rat body weight). This showed that body weight was not a significant factor contributing to mucosal weight. As such a one way analysis of variance assessing the effect of group on mucosal weight was undertaken. The results suggest that there were significant differences in the mucosal weight of animals between groups. It should be noted that all animals received the same treatment in terms of husbandry prior to euthanasia.

Table 65. Comparison of wet mucosal weights between animals in different groups

Group (n=8)	Mean body weight (g)	Mean mucosal weight (g)	F (3,31)	*p*	LSD
1	420 (± 8.53)	2.90 (± 0.15)[a]			
2	436 (± 12.6)	2.64 (± 0.322)[a,b]	4.83	0.0008	0.3838
3	397 (± 9.26)	2.34 (± 0.274)[b]			
4	401 (± 7.35)	2.27 (± 0.153)[b]			

The numbers in brackets after mean weights are the standard errors for each mean (n=8). Means across groups that have a different superscripted letters are significantly different from each other (α=0.05).

13.1.1 Pre-purification control of diet

As brush border enzyme expression (Tanaka et al., 1998, Tanaka et al., 2008), and activities (Ferraris et al., 1992, Tanaka et al., 1998), were found to be affected by nutrient intake (Tanaka et al., 1998) a comparison of BB enzyme activities, in response to commercial diets, was undertaken, table 67. In order to determine whether there were significantly differences in the activities of BB enzymes with dietary change we compared the enzyme activity of rats eating two commercial diets. There are slight

differences in the composition of Pro lab RMH 3000 diet and the Specialty Feeds meat free rat and mouse diet. Dietary energy in Pro lab diet was provided by 26 % protein, 14 % fat and 60 % carbohydrate whereas the Specialty feeds diet has 23 %, 12 % and 65 % respectively. The composition of ingredients also differs, which would account for the differences in activities found.

Table 66. Differences in the brush border enzyme activity of animals fed two different diets (n=19: N=9 Pro Lab diet; N=10 Specialty Feeds diet)

Enzyme	Pro Lab diet (mean U/U[#])	Specialty feeds diet (mean U/U)	LSD	F (1, 17)	*p*
Sucrase	0.60	0.92	0.2359	8.33	0.010
Lactase	0.14	0.25	0.0775	9.16	0.008
Alkaline phosphatase*	3.83	3.79	1.062	0.01	0.934
Dipeptidylpeptidase IV	0.70	1.18	0.515	3.96	0.063

* Subject 7 excluded
# U/U: µmol per min (U) of enzyme activity per U of maltase activity.

Unable to justify standardising enzyme activity against protein or mucosal scraping weight the activity was standardised against the enzyme with the most consistent enzyme activity. Hence, activity was expressed as μmol/min (U) per µmol/min of maltase activity (U), *i.e.* U/U. Result show that there were significant differences in lactase and sucrase activities between the Pro lab diet and Specialty feeds diet. The diets consist of 60 % and 65 % dietary energy from carbohydrate respectively, which may contribute to this difference. The other two enzymes ALP and DPPIV were not significantly different between diets. A power analysis on this data suggests that in order to be 80 % sure of detecting a significant difference in the enzyme activities of animals receiving these diets 22-211 animals. This data is shown in table 67. Having to use such large numbers of animals to detect a significant difference would make this work untenable. This work demonstrated that there were significant inter-animal variations in enzyme activities. In

order to minimise inter-animal differences the mucosal scrapings of animals were pooled (n=8). This number was chosen solely on my ability to process the intestinal scrapings within a timely fashion.

Table 67. Power Analysis based on the data in table 66.

Diet/enzyme	Sucrase	Lactase	ALP	DPPIV
	Number of rats			
Pro Lab	42	130	171	211
Specialty Feeds	22	35	35	77

In order to control nutrient intake rats were changed from a commercial diet (Pro Lab or Specialty Feeds) to a standardised experimental diet: AIN93G (Reeves et al., 1993) at 8 weeks of age. This diet was manufactured, pelleted and dried in-house before being stored in sealed bags at -20 ^{O}C. The diet was used within 3 months as per recommendations (Reeves et al., 1993). Animals were fed *ad libertum* until the night before sampling.

Removal of small intestinal mucus

There is a viscous layer of mucus, up to 480 µm thick, overlaying small intestinal mucosa (Atuma et al., 2001). The role of this mucus is to protect the underlying mucosa from physicochemical damage; it is therefore firmly attached to the underlying mucosa and cannot be rinsed off during step 1 of the purification process, resulting in mucosal scrapings with high mucus content. Sulphydryl reducing agents, such as dithiothreitol (DTT), are known to dissolve mucus (Cone, 2009) so a trial was undertaken to look at the effect of DTT on enzyme activity. Results suggest that BB maltose hydrolysing activites are inhibited 2-4 fold when DTT is used (results not shown). In addition, the purification process was not made easier by the dissolution of mucus, so this addition was rejected.

13.1.2 Stability of the enzymes during storage

It was important to ensure that BB enzymes were stable following purification so time series experiments were undertaken to examine enzyme activity over time under general storage conditions. Figure 64 follows the activity of BB maltase in the BBMV sample from one rat over the course of (82 days) 12 weeks. The GOPOD method, of glucose determination, was used to assay maltolysis (section 3.5.3). Maltose was present at 0.5 mM and the concentration of BBMV was 4 mg/ml (effective concentration of homogenate prior to purification). Where treatments contained NaA it was used at 0.05 %. Results suggest that there was an initial drop in the activity of maltase over the first month that is independent of storage method. The sample frozen (with NaA) and defrosted on the day of BBMV harvest lost greater activity during that process than any of the other samples. This trial only examined the maltolytic activity of the BBMV fraction from one rat a further study was undertaken looking at activity during the first few days after harvest.

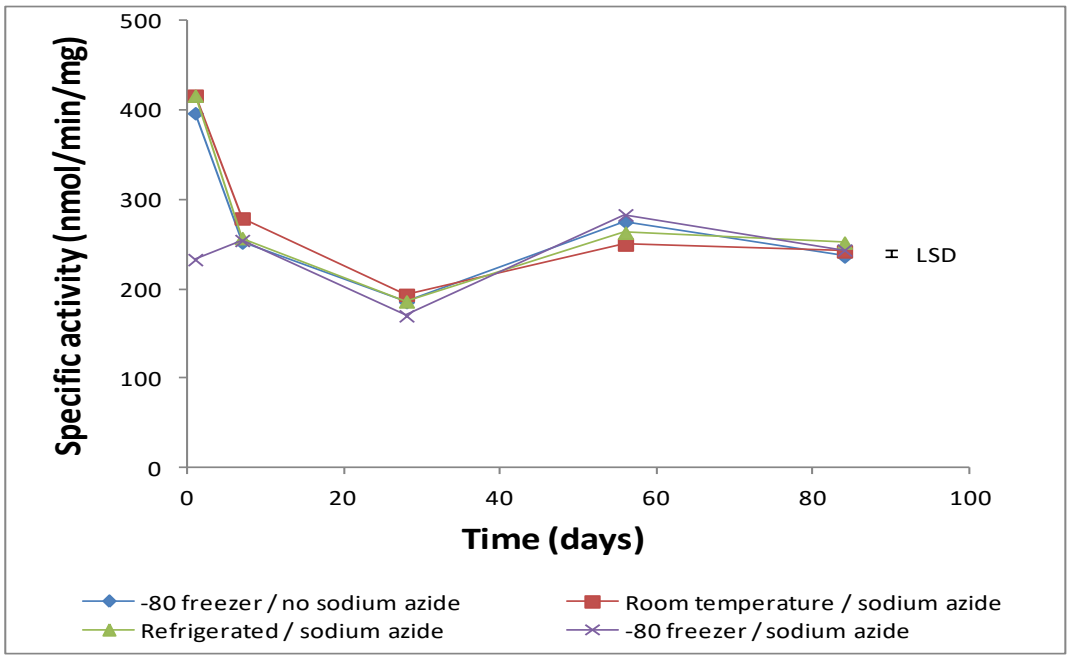

Figure 64. Time series showing the hydrolysis of maltose

Hence the next study involved following the enzyme activity of 4 enzymes from 8 animals following the first 5 days after tissue harvest. The samples were also assayed 1 year later to check ongoing activity. The enzymes assessed were maltase (0.5 mM), sucrose (23 mM), lactose (23 mM) and ALP (0.5 mM). The results are shown in tables 68 and 69. Results suggest that different enzymes respond differently to storage. For example there was no significant degradation of sucrase activity over time or with storage conditions or treatment. Whereas ALP activities were significantly effected by time and storage conditions. Hence, it is important to ensure that when BBMV fractions are collected that they are assayed under the same conditions as previous batches. These results also reinforce the idea that the BBMV provides stability to the enzymes. The room temperature sample is evidence of this. Maltase, sucrase and lactase maintained activities 365 days after harvest

Table 68. The effect of storage conditions on enzyme activity over time.

Enzyme	Treatment #	Time 0 (days)	Time 1 (days)	Time 3 (days)	Time 5 (days)	Time 365 (days)
				Mean (nmol/min)		
maltase	1	0.589	.	.	.	.
maltase	2	.	0.552^{a}	0.655^{b}	0.644^{b}	0.460^{a}
maltase	3	0.581^{a}	0.516^{a}	0.682^{b}	0.587^{a}	0.440^{c}
maltase	4	0.551^{a}	0.535^{a}	0.698^{b}	0.576^{a}	0.215^{c}
maltase	5	.	0.616^{a}	0.645^{a}	0.593^{a}	0.456^{b}
sucrase	1	0.22	.	.	.	.
sucrase	2	.	0.217	0.167	0.225	0.155
sucrase	3	0.201	0.168	0.186	0.186	0.208
sucrase	4	0.194	0.177	0.216	0.190	0.210
sucrase	5	.	0.187	0.182	0.189	0.160
lactase	1	0.020^{1}	.	.	.	.
lactase	2	.	$0.010^{a,1}$	$0.015^{a,1}$	$0.009^{a,1}$	$0.013^{a,1}$
lactase	3	$0.014^{a,1}$	$0.017^{a,1}$	$0.009^{a,1}$	$0.005^{a,,1}$	$0.023^{a,1}$
lactase	4	$0.011^{a,1}$	$0.004^{a,1}$	$0.008^{a,1}$	$0.004^{a,1}$	$0.049^{b,2}$
lactase	5	.	$0.012^{a,1}$	$0.009^{a,1}$	$0.003^{a,1}$	$0.044^{b,2}$
ALP	1	1.58^{1}	.	.	.	.
ALP	2	.	$2.250^{a,1}$	$1.367^{b,1}$	$1.202^{b,1}$	$0.351^{c,1}$
ALP	3	$1.024^{a,1}$	$2.447^{b,1}$	$1.681^{c,1}$	$2.233^{d,2}$	$1.12^{e,2}$
ALP	4	$1.734^{a,1}$	$2.252^{b,1}$	$1.533^{a,b,1}$	$2.342^{a,b,2}$	$1.077^{a,2}$
ALP	5	.	$1.355^{a,2}$	$1.584^{a,1}$	$1.584^{a,1}$	$0.176^{b,1}$

Means across time, within each treatment group that have a different superscripted letters are significantly different from each other. Means across treatments within time groups that have different superscripted numbers are significantly different from each other (α=0.05). #: 1: Fresh; 2: Fridge + sodium azide; 3:-80°C and sodium azide; 4: -80°C; 5: room temperature + sodium azide.

Table 69. Statistical information for data in table 68

Source of variation	Enzyme	df	F	p	LSD
Storage	maltase	4,28	2.62	0.056	0.05091
Time	maltase	4,97	57.44	<0.001	0.04220
Time*storage	maltase	10,97	4.22	0.002	0.09986
Storage	sucrase	4,28	1.25	0.314	
Time	sucrase	4,98	0.55	0.59	
Time*storage	sucrase	10,98	1.97	0.096	
Storage	lactase	4,28	2.42	0.072	0.007543
Time	lactase	4,97	33.92	<0.001	0.005871
Time*storage	lactase	10,97	5.89	<0.002	0.014365
Storage	ALP	4,28	7.55	<0.001	0.322
Time	ALP	4,98	31.69	<0.001	0.3871
Time*storage	ALP	10,98	2.24	0.072	0.7684

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The Secretion and Action of Brush Border Enzymes in the Mammalian Small Intestine

Diane Hooton, Roger Lentle, John Monro, Martin Wickham, and Robert Simpson

Abstract Microvilli are conventionally regarded as an extension of the small intestinal absorptive surface, but they are also, as latterly discovered, a launching pad for brush border digestive enzymes. Recent work has demonstrated that motor elements of the microvillus cytoskeleton operate to displace the apical membrane toward the apex of the microvillus, where it vesiculates and is shed into the periapical space. Catalytically active brush border digestive enzymes remain incorporated within the membranes of these vesicles, which shifts the site of BB digestion from the surface of the enterocyte to the periapical space. This process enables nutrient hydrolysis to occur adjacent to the membrane in a pre-absorptive step. The characterization of BB digestive enzymes is influenced by the way in which these enzymes are anchored to the apical membranes of microvilli, their subsequent shedding in membrane vesicles, and their differing susceptibilities to cleavage from the component membranes. In addition, the presence of active intracellular components of these enzymes complicates their quantitative assay and the elucidation of their dynamics. This review summarizes the ontogeny and

D. Hooton
Institute of Food Nutrition and Human Health, Massey University, Private Bag 121222, Palmerston North, New Zealand

The New Zealand Institute for Plant & Food Research Limited, Food Industry Science Centre, Batchelar Road, Palmerston North 4474, New Zealand

R. Lentle (✉)
Institute of Food Nutrition and Human Health, Massey University, Private Bag 121222, Palmerston North, New Zealand
e-mail: R.G.Lentle@massey.ac.nz

J. Monro and R. Simpson
The New Zealand Institute for Plant & Food Research Limited, Food Industry Science Centre, Batchelar Road, Palmerston North 4474, New Zealand

M. Wickham
Reacta Biotech Limited, Manchester, United Kingdom

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Author(s) full name(s): Hooton, DF, Lentle RG, Monro J, Wickham MSJ, Simpson RM

Corresponding Author's name, address, affiliation and e-mail: Prof. Roger Lentle, Institute of Food Nutrition and Human Health, Massey University, Palmerston Noth, New Zealand, R.G.Lentle@massey.ac.nz

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